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Differential mucosal gene expression modulates the development of murine colitis

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Differential mucosal gene expression modulates the development of murine colitis

by

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DEDICATION

This dissertation is dedicated to:

My daughter Zoe Liu, who was two years old on December 16, 2007;

And my son Gavin Liu, who will be born on February 2008;

And my wife Jun Jiang.

You give purpose and meaning to my life

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LIST OF ABBREVIATION

Abbreviation	**Full names**
ANOVA	Analysis of variance
AP-1	Activator protein-1
APC	Antigen presenting cell
ASF	Altered Schaedler's flora
B. hyodysenteriae	*Brachyspira hyodysenteriae*
B3galt5	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 5
Bcl3	B-cell leukemia/lymphoma 3
BrdU	5-bromo-2-deoxyuridine
CAR	Constitutive androstane receptor
(Nr1i3)	(Nuclear receptor subfamily 1, group I, member 3)
Ccl5	Chemokine (C-C motif) ligand 5
Ccl8	Chemokine (C-C motif) ligand 8
Ccr10	Chemokine (C-C motif) receptor 10
Ccr5	Chemokine (C-C motif) receptor 5
CD	Crohn's disease
cDNA	Complementary DNA
CDT	Cytolethal distending toxin
Ceacam12	CEA-related cell adhesion molecule 12
Ces1	Carboxylesterase 1
Ces3	Carboxylesterase 3
Chuk(IKKα)	Conserved helix-loop-helix ubiquitous kinase (I-kappa-B kinase-alpha)
Cyp4b1	Cytochrome P450, family 4, subfamily b, polypeptide 1
DAVID	The Database for Annotation, Visualization and Integrated Discovery
DC	Dendritic cells
DPI	Days post infection
DSS	Dextran sodium sulfate
EHS	Enterohepatic *Helicobacter* species
ELISA	Enzyme-Linked ImmunoSorbent Assay
FDR	False discovery rate
Fos	FBJ osteosarcoma oncogene
Foxp3	Forkhead box P3
Fut2	Fucosyltransferase 2
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GBP	Guanylate nucleotide binding protein
GO term	Gene-ontology term
Gzmb	Granzyme B
H&E	Hematoxylin and eosin stain
H. bilis	*Helicobacter bilis*
HLAB27	Human Leukocyte Antigen B27
IBD	Inflammatory bowel disease
Ifi47	Interferon gamma inducible protein 47

LIST OF ABBREVIATION (Continued)

Abbreviation	Full names
IFNγ	Interferon gamma
Map3K14	mitogen-activated protein kinase kinase kinase 14
(NIK)	(NF kappa B-inducing kinase)
IL	Interleukin
Irf4	Interferon regulatory factor 4
ItgaL(Cd11a)	Itegrin alpha L (CD11a molecule)
Itgam (Cd11b)	Integrin alpha M (CD11b molecule)
Itgb2(Cd18)	Integrin, beta 2 (CD18 molecule)
Lcp	Lymphocyte cytosolic protein 1
LFA-1	Lymphocyte function-associated antigen 1
LPL	Lamina propria lymphocytes
Ltbr	Lymphotoxin B receptor
Mcpt	Mast cell protease
Mdr1a	Multi-drug resistance 1a
MLN	Mesenteric lymph nodes
MPO	Myeloperoxidase
Muc2	Mucin 2
NFκb	Nuclear factor kappa B
NFκb1(p50)	Nuclear factor of kappa light chain gene enhancer in B-cells 1, p105 (p50)
NFκb2(p52)	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100 (p52)
Nod2	Nucleotide-binding oligomerization domain containing 2
p65	p65 NF-kappa B
(RelA)	(v-rel reticuloendotheliosis viral oncogene homolog A)
Pparγ	Peroxisome proliferator-activated receptor gamma
PXR	Pregnane x receptor
qPCR	Quantitative polymerase chain reaction
Reg	Regenerating islet-derived
RT-PCR	Reverse transcriptase-polymerase chain reaction
SCID	Severe combined immunodeficiency
SEM	Standard error of measurement
Socs3	Suppressor of cytokine signaling 3
TCR	T cell receptor
TGFβ	Transforming growth factor, beta
TLR	Toll-like receptor
TNBS	Trinitrobenzesulfonic acid
TNF	Tumor necrosis factor
Tnfsf13b (BAFF)	Tumor necrosis factor (ligand) superfamily, member 13b (B-cell activating factor)
Treg	Regulatory T cells
UC	Ulcerative colitis
Ugt8a	UDP galactosyltransferase 8A

CHAPTER 1. GENERAL INTRODUCTION

General Introduction and Dissertation Organization

Inflammatory bowel diseases (IBD), including Crohn's disease and ulcerative colitis, are chronic, relapsing intestinal inflammatory enteropathies and as many as 1.4 and 2.2 million individuals in the United States and Europe, respectively, are suffering from these diseases.[1,7] However, the etiology of IBD remains poorly understood and likely involves complex interactions among gut bacteria, epithelial barrier, host immune responses, and genetic susceptibility.[1] The characterization of mucosal gene expression profiles in different IBD animal models will potentially provide molecular insights into the mechanisms that initiate or perpetuate IBD development. This dissertation will study how differentially regulated mucosal genes, induced by different agents (e.g., mild bacterial pathogen, *Helicobacter bilis* or anti-inflammatory drug, hypoxoside), modulate the development of murine colitis.

The dissertation is composed of five chapters. Chapter 1 is a general introduction which includes the organization of dissertation and literature review. Chapter 2, Chapter 3 and Chapter 4 each consists of a research paper. The first paper, "Mucosal gene expression profiles following the colonization of immunocompetent gnotobiotic C3H mice with *Helicobacter bilis*: a prelude to colitis" is to be submitted for publication in *Inflammatory Bowel Diseases*. The second paper, "Effects of *Helicobacter bilis* colonization on murine colitis induced by dextran sodium sulfate" is to be submitted for publication in *Clinical and Experimental Immunology*. The third paper, "Oral treatment with hypoxoside ameliorates *Brachyspira hyodysenteriae*-induced murine colitis" is to be submitted for publication in *American Journal of Physiology - Gastrointestinal and Liver Physiology*. Chapter 5 is a general conclusion including summary of results and discussion of significant findings and recommendations for further research. References of each research paper follow the requirements of the specific journal to which it will be submitted. References of literature review and general conclusion follow the format of the journal *Inflammatory Bowel Diseases*.

Literature Review

Introduction for Inflammation Bowel Diseases

The major forms of idiopathic inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC) are chronic inflammatory disorders of the gastrointestinal tract.[1]

Crohn's diseases vs. ulcerative colitis

Both CD and UC cause inflammation of the bowel (intestine) characterized by symptoms such as bloody diarrhea with mucus, abdominal pain, rectal bleeding. However, there are some important differences in clinical and pathological features between these two forms of IBD.

Crohn's disease, named after American physician Burrill B. Crohn,[2] affect the whole-thickness of the intestinal wall ("transmural") and most frequently cause severe lesions in the ileum, although they can occur at any site along alimentary tract. The inflamed sections can be interrupted by healthy sections. In contrast, ulcerative colitis usually affects the mucosa of intestine ("superficial") and causes severe lesion only in colon. The inflamed sections tend to be continuous.[3]

One of the major histological features of CD is the development of mucosal granuloma, which are composed of compact macrophages, giant cells, and epithelioid cells surrounded by marked infiltration of lymphoid cells, plasma cells, and other inflammatory cells.[4] In contrast, ulcerative colitis is characterized by the formation of crypt abscesses, which are composed of neutrophilic exudate and sloughing and dying cells from the surrounding epithelium.[4]

It is important to differentiate between the two forms of IBD because of the differences in the course of disease and available treatment options. In some cases, however, it may not be possible to tell the difference, in which case the disease is classified as indeterminate colitis.[3]

Epidemiology of IBD

Epidemiological information such as the incidence and prevalence across age, gender, race, geographic regions, or time is likely to provide some clues about the etiology of a given disease or syndrome. With regards to age, the onset of IBD tends to occur in most individuals during the second or third decades of life indicating that some susceptibility factors may accumulate over time. There is no obvious gender difference for IBD patients, although some studies show that females slightly predominate in Crohn's disease while males in ulcerative colitis.[5]

IBD is more likely to occur in blacks or Caucasians people, especially Jewish people, in comparison to individuals of Asian or Hispanic dissent. For example, a North American study evaluating Crohn's disease between 1982 and 1988 reported that the prevalence rates among Hispanic (4.1 per 100, 000) and Asians (5.6 per 100, 000) were much lower than that for Caucasians (43.6 per 100, 000) or African-Americans (29.8 per 100, 000).[6] In addition, familial aggregation of IBD patients has long been recognized. First-degree relatives of affected individuals have a relative risk of 5-fold or greater than those in the general population.[4] These results suggest genetic factors are involved in the development of IBD.

Geographically, the greatest incidence and prevalence of IBD is in highly industrialised Western societies such as those in North America, northern Europe, and the United Kindom. It is estimated that more than 1.4 million and 2.2 million people in United States and Europe, respectively, suffer from this disease annually.[7] Even though there was a rapid increase in incidence and prevalence of IBD during the latter half of the 20th century, the number of IBD patients in the United States and Europe has begun to stabilize. However, in other regions such as Eastern Europe, Asia, and other developing countries, the incidence and prevalence of IBD has begun to increase rapidly, possibly due to environmental or cultural changes associated with diet, lifestyle, and hygienic conditions.[8-10] In addition, other risk factors such as smoking and appendectomy have been associated with incidence of IBD.[11] Collectively, epidemiological studies have suggested that both genetic and environmental factors contribute to the development of IBD.

Diagnosis of IBD

Because the symptoms of IBD such as abdominal pain and rectal bleeding can occur in patients with parasitic infections, celiac disease, or colon cancer, the first diagnostic evaluation of a patient should rule out other causes. This should then be followed by serological and stool tests (e.g., complete blood cell count, electrolyte panel, and fecal occult blood test, stool culture) that can be used to measure the level or severity of inflammation, bleeding, and/or specific bacterial infection. However, the most convincing diagnosis for IBD comes from colonoscopy.[12] In addition, radiologic (X-rays), and pathologic (analysis of tissues) examinations are often required to confirm the IBD diagnosis. Notably, recent advances in serologic markers, endoscopy, and radiology have led to rapid expansion of diagnosis and disease activity assessment of IBD.[13]

Treatment of IBD

The goal of IBD treatment is to control ongoing inflammation including achieving remission (i.e., absence of symptoms) and, once that is accomplished, maintaining remission (i.e., prevention of flare-ups). Surgery is required in many patients with inflammatory bowel disease at some point in their disease. While surgery usually results in an improvement in quality of life, recurrence of disease occurs frequently with reported rates of 5-90% at 1 year, depending on the criteria used.[14] At present, except surgery, there are 5 basic subcategories of medication for the treatment of IBD. (1) Aminosalicylates are compounds that contain 5-aminosalicylic acids (5-ASA). They are the mainstay and first-line drugs for both inducing and maintaining remission in mild to moderate IBD patients, although not all patients respond to these drugs and some severe side-effects occur. It has been shown that 5-ASA is effective for the treatment of ulcerative colitis, whereas its effectiveness for treating Crohn's disease is somewhat controversial.[15] (2) Corticosteroids, which are powerful and fast-acting anti-inflammatory drugs, have been mainly used to achieve short-term remission. Long-term use of corticosteroids is not advised because of undesirable side effects such as immunosuppression. (3) Immunomodulators such asazathioprine and 6-mercaptopurine are immunosuppressive drugs used to maintain remission in Crohn's disease and ulcerative colitis. One of the disadvantages of immunomodulators is that, by suppressing the immune

system, they decrease the body's ability to combat infection. (4) Broad-spectrum antibiotics, such as metroniazole, are also used to treat IBD patients. Aberrant host responses to antigens derived from the intestinal microflora, including commensal bacteria, has been implicated in the etiology of IBD and provides a rationale for the use of antibiotics in the treatment of the disease.[16] However, available studies show that antibiotics are only effective in treating some septic complications in Crohn's disease but are not useful for the treatment of ulcerative colitis.[17] (5) More recently, several biological therapies have been developed to treat cases of IBD that do not respond to the other treatment modalities. Patients with IBD frequently experience relapse and traditional medical treatments are not potent enough to maintain remission over the long-term.[18] Novel therapies such as biological therapies (e.g., monoclonal antibodies) have been developed in recent years with the potential to provide more effective and safe treatments for IBD. In contrast to conventional therapies, most of biological therapies not only reduce the IBD symptoms, but also target the specific disease mechanisms. In this way, these therapeutic approaches have the potential to alter the long-term course of the disease or the underlying immunopathology. Many of the biological therapies include the use of monoclonal antibodies specific for lymphocyte adhension molecules (e.g., alpha4beta7),[19] cytokines (e.g., TNFα, IL-12 and IL-23),[20-22] or growth factors (e.g., granulocyte colony-stimulating factor).[23] Another novel therapy employs the use of helminthes (*Trichuris suis*) to stimulate anti-inflammatory immune responses (i.e., IL-10, Th2).[24] However, the high cost and long-term safety associated with many of the biological therapies still needs to be addressed.[25]

In efforts to develop complementary and alternative medicine (CAM) approaches, plant extracts with anti-inflammatory activities have been used to treat IBD patients and have been shown to prevent or ameliorate colitis in laboratory animals treated with DSS or TNBS.[26-30] These represent a different class of medicinal options for IBD patients. Though the exact components are not completely known, many of these plant extracts, usually as traditional medicine from countries such as China, India or Africa, have been used for centuries for multiple diseases, indicating that they may be safe for human.[26, 31, 32] The anti-inflammatory effects of those plant extracts might be involved in the inhibition of inflammatory mediators, adhesive molecules or oxidative stress.[28-31] For example, boswellic acid, an active component

of oleogum resins from *Boswellia* species could inhibit the production of 5-lipoxygenase and has shown efficacy in some autoimmune diseases including rheumatoid arthritis, bronchial asthma and inflammatory bowel disease with little side effects.[28] Quercitrin, a common antioxidant flavonoid found in vegetables, can be converted into quercetin by gut bacteria and has been shown to inhibit DSS-induced colitis in rats through down-regulation of the NF-κB pathway.[27]

Hypoxoside

Hypoxoside is an example of a botanical extract that has been shown to have anti-inflammatory activities. The ability of this compound to ameliorate colitis caused by *Brachyspira hyodysenteriae* is discussed later in this dissertation (Chapter 4)

Hypoxoside is a diglucosidic compound present in corm of the African medicinal plant *Hypoxis hemerocallidea* (also known as *Hypoxis rooperi*, African Potato), a member of the family *Hypoxidaceae*.[33] This extract has been widely used in southern African as traditional medicine for centuries and it use has been claimed to be an effective remedy/complementary medicine for an array of human aliments such as HIV/AIDS-related diseases, arthritis, diabetes mellitus, cancers, gastric and duodenal ulcers, and urinary tract infections.[34-38] It gained more attention as an alternative medicine for HIV/AID patients following receipt of a strong recommendation from the Ministry of Health of South Africa and 14 other member states of the South African Development Community (SADC).[39] The ingredients of African Potato have been included in some "over-the-counter" medicines.[40]

Rooperol is considered as the major bioactive compound in *Hypoxis hemerocallidea* corm extracts of the African Potato.[42] The primary structure of the pro-drug, hypoxoside, consists of an E-pent-1-en-4-5-carbon unit containing two catechol groups to which the glucose moieties are attached.[41] In the colon, hypoxoside can be converted into rooperol by β-glucosidase produced by members of the gut microflora.[41]

It has been shown that rooperol has anti-inflammatory effects and has potential usefulness in the treatment of inflammatory diseases.[43-46] Rooperol was shown to inhibit the production of TNF-α, IL-1, IL-6, and nitric oxide by endotoxin-stimulated human alveolar macrophages, human blood monocytes, histiocytic cells, and rat alveolar macrophages *in*

vitro.[43] Rooperol decreased the mRNA and protein expression levels of vascular cell adhesion molecule-1(VCAM-1) and nitric oxide synthease (iNOS) in microvascular endothelial cells stimulated by TNF-α or IFN-γ.[44] In addition, rooperol was shown to decrease the mRNA levels of pro-inflammatory cytokines in a human promonocytic cell line and inhibit the transient binding activity of transcriptional factors including NF- κB and AP-1(c-jun/c-fos dimer).[45] Similarly, *H. hemerocallidea* corm extracts were shown to have anti-inflammatory effects on fresh egg albumin-induced acute inflammation in rats.[46] In addition to inhibition of pro-inflammatory cytokines, the anti-inflammatory effects were also proposed to be associated with the inhibition of the 5-lipoxygenase pathway, but not cyclooxygenase.[47] However, one recent *in vitro* study of rooperol showed a significant degree of inhibition of cyclooxygenase-1 and 2.[48] Rooperol was also shown to inhibit the growth of gram-negative bacteria (e.g., *Escherichia coli*) [48,49] and gram-positive bacteria (e.g., *Staphylococcus aureus*)[48]. In addition, rooperol was shown to have significant anti-oxidative activity.[50] Meantime, hypoxoside has not exhibited toxicity during *in vitro* or during clinical trials.[51, 52]

.

Animal models of IBD

Animal models of IBD have played a key role in understanding the mechanisms of intestinal inflammation and in designing new therapeutic strategies for the treatment of IBD patients. Based upon the underlying method of disease induction, there are several broad categories of IBD animal models that have been used to study the mechanisms of disease: gene knockout (KO) (e.g., IL-$2^{-/-}$, IL-$10^{-/-}$, TCR$\alpha^{-/-}$ and Mdr1$a^{-/-)}$, transgenic (e.g., HLA-B27, Stat-4 transgenic), chemical (e.g., DSS, TNBS), adoptive transfer (e.g., $CD4^{+}$ $CD45RB^{high}$ transfer), and spontaneous (e.g., C3H/HejBir, SAMP1/Yit).[53] Based on the cellular and molecular mechanisms associated with the development of IBD, animal models can be placed into one of three types of basic defects: altered innate immunity (e.g., Mdr1$a^{-/-}$, DSS), excessive T cell responses (e.g., IL-$2^{-/-}$, Stat-4 transgenic), or altered regulatory mechanism (e.g., $IL2^{-/-}$, IL-$10^{-/-}$, TGF$\beta1^{-/-}$ and TCR$\alpha^{-/-}$) (Table 1). Other models (e.g., C3H/HejBir, SAMP1/Yit) demonstrate milder defects in more than one component (Table 1).

Table 1. Representative murine colitis models[54]

Model	Altered innate immune response	Excessive effector T-cell response	Altered regulatory mechanisms	Bacterial flora driven	Known defects
Mdr1a$^{-/-}$	√			**Probable**	**Altered epithelial barrier**
DSS	√			**Possible**	**Direct damage to epithelial barrier**
Stat-4 trangenic		√		**Probable**	**Excessive T-cell responses to enteric bacteria**
C3H/HeJBir	√	√		**Yes**	**Impaired responses to TLR ligands increased bacterial reactive T cells**
SAMP1/Yit	√	√		**Yes**	**Epithelial cell defects; expanded B-cell population; increased activated T cell**
IL-2 deficient			√	**Yes**	**Decreased regulatory T cell**
IL-10 deficient	√		√	**Yes**	**Lack Tr1 activity; lack TGFβ signaling**
TGFβ1 deficient	√		√	**No**	**Decreased regulatory T cells**
TCRα deficient			√	**Yes**	**Loss of a regulatory B cell**
CD4^{+}CD45RB^{hi} transfer			√	**Yes**	**Decreased regulatory T cells**

Mdr: Multi-drug resistance; DSS: Dextran sodium sulfate; Stat-4: Signal transducer and activator of transcription 4
IL: Interleukin; TGF: Transforming growth factor; TCR:T- cell receptor
Modified from Elson CO, et al, 2005.

IBD animal models with altered innate immunity

1. Mdr1a$^{-/-}$ mice. These mice lack the multiple drug resistance genes for P-glycoprotein 170 (pg170), a xenobiotic transporter normally expressed in multiple tissues including intestinal epithelial cells. Pg170 is involved in detoxification processes and maintenance of the intestinal barrier.[55] These mice spontaneously develop colitis around 12 weeks of age.[56] Polymorphisms in *Mdr1* have been shown to be strongly associated with ulcerative colitis.[57,58] Although pg170 is also expressed in lymphocytes, hematopoietic cells, and in epithelial cells of other tissues, it has been shown that the intestinal epithelial cell defect is the principle cause of colitis.[59,60]

Helicobacter bilis, a mild pathogen, has been shown to accelerate colitis development in Mdr1a $^{-/-}$ mice.[61,62] In comparison to wild-type mice colonized with *H. bilis*, Mdr1a$^{-/-}$ mice colonized with *H. bilis* were characterized by the upregulation of mucosal *IFN-γ*, *IL-10* gene expression and increased lamina proprial cellularity with expansion of CD4 and CD8 T cells and B cells. In addition, there was also increased antigen-specific recall responses by T cells recovered from the mesenteric lymph node (MLN) when stimulated with soluble *H. bilis* antigens. These proliferative responses were accompanied by the elaboration of IFN-γ, TNF-

α, and IL-10.[61] These results indicated that, upon *H. bilis* colonization, the defective mucosal barrier could lead to the disruption of gut homeostasis and activation of adaptive immune responses.

2. Dextran sodium sulfate (DSS)-induced colitis. The addition of DSS into the drinking water can induce colitis in mice, rats, and hamsters accompanied by clinical signs such as bloody diarrhea, and weight loss. Macroscopic and microscopic changes include shortening of the colon, neutrophilic infiltration, and epithelial changes including fibrosis, crypt loss, goblet cell hyperplasia, and focal ulceration.[63-65] Exposure to DSS can induce either acute colitis that occurs during the administration of DSS, or chronic colitis that occurs a short time after the cessation of DSS treatment. Colitis was shown to be present in SCID mice treated for short periods with DSS, suggesting T cells, B cells, and NK cells are not essential in the acute phase of DSS colitis.[66,67] However, chronic colitis is considered to be caused by activation of lymphocytes mediated by cytokines secreted from activated macrophages.[63] The pathogenesis of DSS-induced colitis is widely considered to be caused by the direct damage to the intestinal epithelial cells.[63] The initiating events induced by DSS seems to decrease epithelial proliferation[68] and/or increase epithelial permeability (e.g., defective tight-junction proteins)[69, 70]. The change in epithelial barrier function then allows lumen antigens to activate the resident immune cells such as macrophages facilitating the development of colonic inflammation[71].

The association of enteric bacteria with DSS-induced colitis is well described. Some bacterial species (e.g., *Bacteroides, Enterobacte*r and *Clostridium*) have been shown to significantly increase after acute DSS-induced colitis. [63] The administration of antibiotics (e.g., metronidazole or the combination of metronidazole and ciprofloxacin) protected rodents from acute DSS-induced colitis, [72] suggesting that bacteria or the host responses to bacteria can contribute to the development of colitis. Another study demonstrated that antibiotics with narrow specificities (e.g., metronidazole) could prevent the DSS-induced colitis but had no therapeutic benefit once colitis was established.[73] On the other hand, broad-spectrum antibiotics (e.g., vancomycin-imipenem) were able to more effectively prevent DSS-induced colitis, as well as treat colitis.[73] This indicates that different subsets of bacteria have different propensities to initiate or perpetuate the development of DSS-induced colitis.

In addition, *Staphylococcus aureus* enterotoxin B has been shown to aggravate enteric inflammation in mice recovering from DSS-induced colitis, indicating some bacterial products can perpetuate colitis[74]. In this dissertation, the effects of *Helicobacter bilis* on the DSS-induced colitis will be discussed in Chapter 3. However, the exact roles of bacteria in DSS-induced colitis is still controversial and it has been reported that DSS-induced colitis could occur in germ-free environments.[75, 76]

IBD animal models with excessive T cell responses

1. STAT-4 transgenic mice. STAT-4 (Signal transducer and activating transcription 4) is essential in the signal transduction of the prototypical Th1 cytokine, IL-12.[77] *Stat-4* transgenic mice develop chronic transmural colitis characterized by infiltrates of $CD4^+$ T lymphocytes which produce predominantly TNFα and IFN-γ.[78]

2. C3H/HejBir mice. In these mice, colitis occurs spontaneously at 3 to 4 weeks of age and resolves by 10 to 12 weeks of age.[79] The colitic lesions of these mice are characterized by ulcers, crypt abscesses, and regeneration of epithelium, but not thickening of the intestinal wall or formation of granulomas.[79] Bacterial antigen-specific $CD4^+$ T cells have been shown to play a key role in the mucosal inflammatory response of C3H/HejBir mice indicating a pivotal role of bacteria or bacterial products in the induction of colitis. [80] In addition to the excessive T cell responses, C3H/HejBir mice display impaired innate immune responses which also contributes to the increased susceptibility to colitis.[81]

3. SAMP1/Yit mice. These mice spontaneously develop Crohn's disease-like intestinal inflammation characterized by a discontinuous lesion localized to the terminal ileum that is heavily infiltrated with T cells, neutrophils, and macrophages.[82] The localized transmural ileitis is a unique feature not shared with other mouse models of IBD.[83] The inflammatory lesions of SAMP1/Yit mice develop by 10 weeks of age and the host immune response is characterized by a Th1-type profile.[84, 85] The induction of SAMP ileitis has been shown to be mediate by aberrantly activated $CD4^+$ T cells and B cells.[86] However, increased intestinal epithelial permeability has been shown to be the primary factor influencing the increased susceptibility of SAMP1/Yit mice to the development of ileitis.[87]

IBD animal models with altered regulatory mechanisms

1. IL-$2^{-/-}$ mice. Approximately 50% of $IL2^{-/-}$ mice develop severe autoimmune diseases characterized by splenomegaly, lymphadenopathy, and autoimmune hemolytic anemia and die between 4 and 9 weeks of age. The remaining mice uniformly develop pancolitis between 6 and 15 weeks of age.[88] IL-2 is a critical T cell growth factor *in vitro*, but predominantly mediates immune regulation *in vivo*.[89] Although IL-2 is dispensable for the development of regulatory T cells in thymus, it plays an essential role in maintaining peripheral tolerance by promoting the growth and survival of regulatory T (Treg) cells.[90] IL-$2^{-/-}$ mice failed to develop $CD4^{+}CD25^{+}$ Treg subset in the peripheray.[91]

2. IL-$10^{-/-}$ mice. IL-10 is an important immunoregulatory cytokine produced by various types of cells including Treg cells, B cells, dendritic cells, and macrophages. It down-regulates the function of Th1 cells, NK cells, and macrophages. Mice deficient in IL-10 develop intestinal inflammation with age.[92] IL-$10^{-/-}$ mice lack Tr1 cell activity, as well as TGFß/Smad signaling, and fail to inhibit pro-inflammatory gene expression in intraepithelial lymphocytes (IELs).[93] Similar to IL-$2^{-/-}$ mice, IL-$10^{-/-}$ mice display a Th1 cytokine pattern (e.g., elevated IFN- γ and IL-12).[94] Recently, IL-$10^{-/-}$ mice have also been shown to produce high levels of IL-17 derived from IL-17–producing effector T cells, termed Th17.[95] However, one recent study showed that colitis in IL-$10^{-/-}$ mice was dependent on toll-like receptor (TLR) signaling while disease in IL-$2^{-/-}$ mice is not TLR-dependent, indicating a fundamental difference in the underlying mechanisms causing colitis in these two models.[96]

3. TCR$\alpha^{-/-}$ mice. TCR $\alpha^{-/-}$ mice spontaneously develop inflammation and hypertrophy over the entire length of the colon by 16 weeks after birth.[97] Hyperplasia of the colonic epithelium, occasional crypt abscesses and goblet cells and infiltration of lymphocytes, plasma cells, and neutrophils were also observed.[98] In contrast to the Crohn's disease-like colitis observed in *IL-$2^{-/-}$* and *IL-$10^{-/-}$* mice, the colitis in *TCR$\alpha^{-/-}$* mice resembles ulcerative colitis and is characterized by a Th2 cytokine profile.[99] However, regulatory B cells have been shown to play a critical role in regulating pathogenic T cells ($CD4^{+}$ TCRβ^{+} T cells).[99]

4. CD45RB transfer model. Murine $CD4^{+}CD45RB^{high}$ T cells have been characterized as naive T cells. Transfer of $CD4^{+}CD45RB^{high}$ T cells, but not total $CD4^{+}$ T cells or

$CD4^+CD45RB^{low}$ T cells, into SCID or $Rag^{-/-}$ recipient mice results in the induction of colitis four to eight weeks later.[100] The disease in this model is progressively unremitting and eventually results death. The histological features of disease caused by the transfer of naïve T cells are similar to that observed in other colitis models and are limited to the intestine, especially the colon.[101] Conclusions drawn from this model suggests that normal, naïve $CD4^+$ T cells contain pathogenic cells that can initiate intestinal inflammation and that inflammation is prevented by the presence of Treg cells.

Notably, the presence of commensal bacteria is indispensible for the development of colitis in most of these models (Table 1). This is one of the most convincing pieces of evidence that support the etiological role of immune responses to commensal bacteria in IBD development.

There are other colitis models that are less frequently used by to study IBD. Colitis model induced by *Citrobacter rodentium* is one example. *Citrobacter rodentium* is an attaching and effacing pathogen and able to adhere to intestinal epithelium.[102] Infection of mice with *C. rodentium* results in a breach of the colonic epithelial barrier that result in the induction of a vigorous Th1 inflammatory response and colitis.[103]

Conclusions from IBD animal models

There are many animal models for IBD now, including what was described above and were not yet discussed. These IBD animal models have revealed that a variety of alteration of immune responses, including innate or adaptive immunity or immune regulatory mechanisms, can result in intestinal inflammation similar to human IBD. The recurring themes derived from these animal models include: (1) host genetic background modulates the disease activity; (2) presence of gut flora including commensal bacteria provides the constant antigenic stimuli driving chronic colitis; (3) chronic inflammation is T cell mediated; and (4) defective epithelial barrier function and alteration of immune regulation can induce the development of colitis. Notably, not all the gut bacteria have the same effect on the development of colitis[73]. In Chapter 3 of this dissertation, the ability or propensity for *Helicobacter bilis* to exacerbate the development of DSS-induced colitis will be discussed.

Within the intestinal mucosa, the aspects of the immune system that are relevant to the development of IBD are comprised of three functional categories including innate immunity (e.g., epithelial barrier function), adaptive immunity (e.g., effector CD4 T cells) and immune regulatory mechanisms (e.g., regulatory T cells). Severe impairment of a single category can lead to disease as has been shown in many animal models of IBD (e.g., Mdr1a$^{-/-}$ mice).[56]However, mild defects in several categories can also lead to disease (e.g., C3H/HejBir mice and SAMP1/Yit mice).[79, 82] These observations fit the "multiple-hit hypothesis", which postulates that defects in two or more components of intestinal mucosal immune systems are required for the development of IBD in immunocompetent hosts.[54] Although alteration of one or several categories of mucosal immunity may contribute to IBD development, the specific mechanisms by which interaction of those categories maintains gut homeostasis or results in intestinal inflammation are complex and not yet completely understood.

Development of Inflammatory Bowel Diseases

Currently, the precise etiology of IBD remains unknown. However, several factors have been considered to contribute to the development of IBD. They include genetic susceptibility, immune responses, luminal antigen/adjuvant and environmental factors.[1] The most widely held hypothesis is that IBD is induced by the dysregulation of immune responses, innate and/or adaptive, to gut commensal bacteria in genetically susceptible hosts.

Gut mucosal tolerance/homeostasis

It is estimated that human gut harbors about 10^{14} total bacteria that include about 300 to 500 species or strains.[104] The mammalian intestinal mucosa comprises the largest lymphoid tissue in the body and is often referred to as gut-associated lymphoid tissue. However, intestinal cells associated with healthy mucosal surfaces are hyporesponsive to the huge antigenic load created by the resident bacterial flora and dietary constituents. This unresponsiveness is commonly referred to as "mucosal tolerance".[105]

The underlying mechanisms, by which mucosal tolerance is maintained, are incompletely understood and may involve regulation of the complex interactions between luminal bacteria, epithelial cells, and lamina proprial cells.[106]

Luminal commensal bacteria play important roles in the development of the mucosal immune system and immune responses. This can be demonstrated in germ-free mice that do not have a resident bacterial flora. In comparison to conventional mice, germ-free mice have smaller Peyer's patches, have fewer intraepithelial lymphocytes, have reduced cytokine production, have reduced serum immunoglobulin levels, and are more susceptible to infection.[107] In addition, the presence of a commensal flora facilitates the induction of protective, structural, and metabolic benefits for the host epithelium. Following the colonization of germ-free mice with a commensal bacterium (e.g., *Bacteroides thetaiotaomicron)*, the expression levels of multiple host genes involved in nutrient uptake, metabolism, angiogenesis, mucosal barrier function, and the development of enteric nervous system were increased.[108]

Gut commensal bacteria seem to be important in maintaining mucosal homeostasis. It has been shown that recognition of commensal bacteria through Toll-like receptors and activation of Myd88 signaling is critical for protection against mucosal injury.[109] Commensal bacteria have been demonstrated to limit mucosal NF-κ B signaling by inhibition of epithelial proteasome function,[110] degradation of I-κB[111] and nuclear export of NF-κ B subunit p65 through a peroxisome proliferator-activated receptor (PPAR) γ-dependent pathway.[112]

As a single layer of cells between luminal bacteria and mucosal lymphoid tissues, the intestinal epithelial cells play a vital role in maintaining mucosal homeostasis. Interdigitated epithelial cells fortified by tight junction proteins such as occludin and claudin serve to maintain the physical barriers in healthy individuals.[113] Mucus covering the surface of epithelial cells also contributes to mucosal homeostasis. For example, $Muc2^{-/-}$ mice spontaneously develop colitis[114] and mice deficient in the gene encoding core 3-derived O-glycans, an important component of mucin core protein, were more susceptible to experimental colitis in comparison to wild-type mice.[115] A defective mucus layer, both in thickness (e.g., absence of mucus in severely inflamed mucosa) or composition (e.g., abnormal glycosylation of mucin) has been reported in IBD patients.[116] In addition,

paneth cells, a special epithelial cell type in small intestine, can secret defensins to combat against the luminal bacteria.[117]

Within the intestinal lamina propria, there are various types of immune cells including T cells, B cells, macrophages, dendritic cells (DCs), granulocytes, mast cells, NK cells, and NK-T cells. Among them, DCs are key cells in maintaining mucosal homeostasis. Dendritic cells can directly sample bacteria via opening the tight junctions between epithelial cells and extending dendrites into the intestinal lumen.[118] In addition, DCs express the whole set of pattern-recognition receptors and can differentiate between the pathogenic and commensal bacteria.[119] The function of DCs is regulated by their location, number, and maturation state. In healthy mucosa, DCs display immature or non-inflammatory phenotypes that are maintained by the paracrine activity of thymic stromal lymphopoeitin (TSLP) factors released from intestinal epithelial cells.[120] More importantly, with the aid of retinoic acid and TGF-β, a subpopulation of gut DC ($CD103^+$) appear to convert peripheral $CD4^+$ T cells into $Foxp3^+$ Treg cells, which also contribute to the maintenance of mucosal homeostasis.[121] In addition, a more recent study reported that a population of $CD11b^+F4/80^+CD11c^-$ macrophages also involved in the induction of $Foxp3^+$ Treg cells in lamina propria.[122]

Collectively, the complex interaction between commensal bacteria, intestinal epithelial cells and lamina proprial immune cells (e.g., DCs, Treg cells) controls the balance between effector and regulatory immune subpopulations through tightly regulated cytokine networks in order to effectively maintain mucosal homeostasis in healthy individuals. Other immune effector cells, such as granulocytes, mast cells, natural killer cells, natural killer T cells, and macrophages reside in their individual tissue compartments and intracellular inhibitory mechanisms prevent the production and/or release of inflammatory mediators that prevent the generation of tissue damage.

Development of IBD

Disruption of gut homeostasis and development of IBD are commonly hypothesized to be the consequence of dysregulation of immune responses to commensal bacteria in genetically susceptible hosts.[1]How and why individuals generate inappropriate host immune responses to commensal bacteria, thus, initiating and perpetuating the development of IBD, is

not fully understood. In other words, what are the host susceptibility factors which contribute to pathogenesis of IBD? In this regard, recent studies of IBD pathogenesis have provided some clues that address how IBD is initiated or perpetuated.

Alteration of mucosa barrier functions.

Intestinal epithelial cells form a physiological barrier between luminal bacteria and the lamina proprial immune cells. Firstly, decreased mucosal resistance and increased permeability has been reported for intestinal tissue recovered from IBD patients and can be used to predict relapse during clinical remission.[123,124] Changes in mucosal permeability are also demonstrable in some first-degree relatives of Crohn's disease patients.[125] The increased permeability might be caused by the changes in expression and distribution of tight junction proteins such as claudin 2, 5, or 8 in patients with active Crohn's disease.[126] Recently, it has been shown that the primary defect of SAMP1/Yit mice, a spontaneous colitis model, is the presence of a leaky intestinal epithelial barrier that was correlated with the altered gene expression of the tight junction proteins claudin-2 and occludin.[87]

Secondly, intestinal epithelial cells also participate in the process of detoxification and biotransformation of drugs and xenobiotics.[127] Loss or dysregulation of detoxification and biotransformation of intestinal epithelial cells has been shown to contribute to the initiation and progression of IBD.[127] A set of genes involved in phase 1, phase 2, and cellular efflux transporters of detoxification processes were coordinately downregulated in the colon of patients with ulcerative colitis.[128] Those downregulated genes are the target genes of nuclear receptor PXR(pregnane x receptor). [128] Recently a PXR polymorphism was identified to be strongly associated with IBD susceptibility.[129] Downregulation of detoxification genes were also found in IBD animal models such as DSS-induced colitis and TCR$\alpha^{-/-}$ mice.[130] Mdr1a$^{-/-}$ mice spontaneously develop colitis, further supporting the importance of mucosal detoxification in the initiation and perpetuation of IBD.[56]

With regards to intestinal epithelial cells, the aberrant expression and distribution of pattern recognition receptors including TLRs and Nod2 were also found in IBD patients.[131,132] In healthy individuals, TLR2 and TLR4 were rarely expressed on the apical surface of epithelial cells while TLR3 and TLR5 were constitutively expressed on the

basolateral membranes of epithelial cells.[133,134] However, TLR2 and TLR4 were significantly upregulated but TLR3 is downregulated on epithelial cells of IBD patients.[135]

Aberrant immunoregulation

In intestinal mucosa, DCs play key roles in inducing mucosal tolerance and active immune responses by interacting with T cells in either the mesenteric lymph nodes or the lamina propria.[136] The decision relative to the choice between the induction of active immunity and tolerance will depend on the subpopulation of DCs involved and their activation phenotype, which is influenced by mucosal microenvironment. [136] Therefore, one of the factors contributing to the initiation of IBD is the inappropriate induction of a DC phenotype that facilitates the induction of aberrant immune responses to commensal bacteria.[137] Associated with a loss of mucosal barrier function, activation of DCs could be caused by dysfunctional or exaggerated TLR signaling related to the increase of phlogistic components from the lumen. Expression of TLR2/4 and the activation/maturation marker CD40 on DCs has been shown to be significantly upregulated in IBD patients.[137]

Due to the presence of over-activated DCs and other factors, the progression of IBD development likely involves either an over-production of effector T cells or the lack of appropriate regulation of effector T cells. The loss of central or peripheral tolerance leads to the long term survival of T cells often mediated by defective apoptosis.[138] Evidence of defective T cell apoptosis and prolonged T cell survival has been found in the mucosa of patients with Crohn's disease.[138] As a measure of the role activated T cells play in the pathogenesis of IBD, induction of T cell apoptosis has been used to treat IBD patients.[139]

In addition, an imbalance between mucosal effector T cells and Treg cells might also increase the potential for the development of IBD. In Crohn's disease, naïve T cells have been shown to differentiate into Th1 or Th17 effector T cells, while in ulcerative colitis, Th2 T cells are associated with disease progression/severity.[140] Under a separate set of conditions, T cells can differentiate into Treg cells including naturally occurring $Foxp3^+$ Treg, Tr1, or Th3 cells.[141] The importance of Treg cells in controlling IBD has been convincingly shown in IBD animal models. Mice deficient in IL-2, IL-10, or TGF-β which are all involved in Treg cell development have been shown to spontaneously develop colitis.[88, 92, 142] For

example, TGF-β is a critical factor that serves to determine the balance between pro-inflammatory Th17 T cells and anti-inflammatory Treg cells.[143] Endogenous and exogenous factors that alter the production of TGF-β and other anti-inflammatory cytokines are likely to affect the development of IBD. Induction of SMAD7, an inhibitor of TGF-β signaling, was implicated as a potential underlying mechanisms contributing to colitis in IBD patients.[144] In addition, other Treg cells such as $CD8^+$ Treg cells were described in IBD and found to be reduced or absent in cells recovered from the lamina propria of IBD patients.[145] However, in contrast to murine studies, most human IBD studies indicate that immune activation is driven by aggressive innate or T-cell responses, rather than defective immunoregulatory functions.[1]

It is clear that multiple factors and complicated interactions associated with epithelial cell barrier function or aberrant immunoregulation play central roles in initiating and perpetuating the development of IBD. Consistently, several genes involved in epithelial barrier functions including *Mdr1a, Slc22a4,* and *Slc22a5* have been identified to be IBD susceptible genes.[146] In addition, a variant of IL-23R, a receptor expressed on the surface of Th17 T cells, has been strongly associated with the reduced risk of IBD[147] suggesting that IL-23R-mediated adaptive immunity is also critical in IBD development. So the identification of IBD susceptible genes reiterates the importance of epithelial barrier functions and immune responses in the development and maintenance of IBD.

Following the initiation of IBD, there are some common or secondary events that precede the development of intestinal inflammation. First, activation of immune responses correlates with the upregulation of chemokines, chemokine receptors, and adhesive molecules leading to infiltration of different immune and inflammatory cell types. Up-regulation of chemokines and chemokine receptors has been described in a series of studies examining clinical and experimental colitis.[148] The major chemokine and chemokine receptors associated with the onset of IBD included CXCL8/IL-8, CCL5/RANTES, CCL2/MCP-1, CCL6/MCP-3, CCR5, CCR2, CCR1 and were involved in the infiltration of inflammatory cells such as neutrophils, macrophages, and T cells.[149] Notably, $\alpha4\beta7^+CCR9^+$ T cells, which can be effector T cells, are specifically recruited into the intestinal lamina propria, especially to the small intestine.[150] The CCL5-CCR5 chemokine axis was identified

to be involved in preferential recruitment of FoxP3$^+$ Tregs into inflamed small intestine in SAMP1/YP mice, which prevents further exacerbation of chronic inflammation in the intestine.[151]

Chemokines and chemokine receptors are crucial inflammatory mediators of IBD and contribute to the pathogenesis of IBD.[152] It has been reported that the use of chemokine or chemokine receptor antagonists as well as monoclonal antibodies specific for adhesion molecules can be used to treat IBD patients.[153,154] For example, antibodies against the α4 chain[155] or the α4β7 integrin attenuated the severity of IBD.[156] In addition, the influx of inflammatory cells into the mucosa results in the release of many metabolic and inflammatory mediators including cytokines, chemokines, nitric oxide, oxygen radicals, prostaglandins, leukotrienes, histamine, proteases, and matrix metalloproteinases, which eventually induces tissue damage.

Mucosal gene expression profiles in IBD

Inflammatory bowel diseases are idiopathic, multigenic disorders involving a complex interaction among genetic, environmental, and immunological factors.[157]Analysis of a single or select few genes is not likely to provide an accurate picture on how and why IBD develops. Microarray technology provides a novel and powerful tool that can be used to define the changes in the large-scale transcriptional profile and help to understand the complex molecular interactions associated with acute and/or chronic IBD.

To date, multiple studies have utilized microarray technology to define mucosal genes expression profiles in IBD patients and/or animal models.[158-163, 128,130] The methods and conclusions drawn by these studies vary due to that different microarray types, statistical methods, tissues/cell population, and stages of disease were employed. However, they do provide a broad picture regarding the changes in mucosal genes expression during the development of IBD. More importantly, several previously known and unknown groups of differentially expressed genes were identified by microarray analysis of intestinal samples collected during the development of IBD.[164]

(1) Genes that mediate inflammation.

This group of genes includes cytokines, chemokine/chemokine receptors, complement components, transcription factors or adaptor molecules in signal transduction. Predictably, this was the most strongly and consistently upregulated group of genes in both IBD patients and animal models. For example, upregulation of genes encoding IL-1ß, IL-6, IL-8, lymphotoxin-β, IL-1 receptor antagonist (IL-1RA), and cyclooxygenase-2 (Cox2) were reported in tissue samples recovered from inflamed colon in comparison to samples from sections of non-inflamed mucosa collected from patients with ulcerative colitis.[158] Another study summarized a series of genes that are known to change transcriptional activity in IBD, including *TNFα, IFN-γ, Ltβ, IL-6, IL-16, IL-18R1, IL-22, CCR2, CCR7, CCL2, CCL3, CCL4, CCL5, CCL7, CCL11, CCL17, CCL20, CXCR3, CXCL1, CXCL5, CXCL10*.[163] The up-regulation of these genes reflected the induction of a non-specific inflammatory reaction characteristic of both Crohn's disease and ulcerative colitis. Interestingly, most differentially expressed genes encoding cytokine or chemokine/chemokine receptors are detected in the CD45RB^{high} transfer model of colitis, in comparison to TNBS and DSS-induced colitis, suggesting that the CD45RB^{high} transfer model better resembles human IBD.[163]

(2) Genes that inhibit inflammation.

Genes encoding anti-inflammatory molecules are commonly upregulated in inflamed tissue, possibly providing a mechanism of negative feedback that facilitates tissue repair or restitution. For example, indoleamine 2, 3-dioxygenase (IDO) is a tryptophan-catabolizing enzyme that has been shown to be potent inhibitor of T cell responses.[165] Over expression of *IDO* was reported in tissues samples recovered from patients with either form of IBD and in epithelial cells recovered from colitic mice using either the DSS-induced or TCRα$^{-/-}$ colitis models.[158, 166, 130] Suppressor of cytokine signaling 3(SOC3) plays an important role in negative regulation of cytokine signaling and suppression of colitis.[167] Expression levels of *Socs3* were significantly upregulated in colonic epithelial cells of IL10$^{-/-}$ and TCRα $^{-/-}$ mice, in SCID mice used in theCD45RB^{high} transfer model, and in mice with DSS-induced colitis.[130] Another study identified five upregulated inflammatory signature genes (*MRP14, GROγ and SAA1, TIMP1 and Elafin*) in colonic tissue recovered from patients with ulcerative

colitis; it should be noted that two of these genes (*TIMP1 and Elafin*) have anti-inflammatory effects.[160]

(3) Genes that promote cell growth/apoptosis.

The Reg gene family includes regenerating islet-derived 1A (*Reg1a*), *Reg1b, Reg3b,* and *Reg3g* and has been shown to stimulate the proliferation of pancreatic cells and gut epithelial cells.[168, 169] The strong upregulation of Reg gene family has been shown in ulcerative colitis,[158] Crohn's disease,[159] and various IBD animal models.[130] Interestingly, in DSS-induced colitis, Reg3g was only detectable in the acute stages of inflammation (e.g., at 4 days) as opposed to the restitution period indicating its role during acute inflammation.[130] The S100 gene family, including *S100a8, S100a9,* and *S100b,* encode inflammatory proteins produced by phagocytes and are involved in apoptosis.[170] Upregulation of the S100 gene family has been also shown in inflamed colon of patients with ulcerative colitis,[158,159], Crohn's disease,[159] and animal models.[163]

(4) Genes that participate in detoxification process.

One study showed that a set of detoxification-associated genes involving phase I (e.g., *Cyp3A4*) and phase II (e.g., *Ugt1a3*) enzymes along with cellular efflux transporters (e.g., *Mdr1a*) were coordinately downergulated in colonic (not ileum) tissues of patients with ulcerative colitis (not Crohn's disease).[128] These changes were correlated with the downregulation of pregnane X receptor (PXR), which was suggested to regulate many of these detoxification-associated genes.[128] The fact that PXR gene locus is strongly associated with IBD predisposition[171] and PXR agonist can ameliorate DSS-induced colitis[172] emphasized the importance of the dysregualtion of detoxification processes in the pathogenesis of IBD. Following the analysis of intestinal epithelial cells, the down regulation of several detoxification genes including cytochrome P450 genes (e.g., *Cyp2c40*), selenium binding protein 1(*Sbp1*), multidrug resistance gene 1a (*Mdr1a*), phenol sulfotransferase-1 (*Pst-1*), carbonic anhydrase IV (*Car4*) has been reported in several animal models of chronic IBD.[130, 173]

(5) Genes that mediate fatty acid metabolism.

Fatty acids play an important role in inflammatory processes as well as in ensuring nutrition and cellular integrity by providing a molecular source for phospholipid synthesis.[174] In comparison to control tissues, a series of genes associated with fatty acid metabolism were downregualted in colonic mucosal tissue recovered from patients with ulcerative colitis.[159] These findings are consistent with an earlier hypothesis that energy deficiency is a significant factor in the pathogenesis of colitis.[175] Another study identified changes in the expression of the liver fatty acid-binding protein (*L-fabp*), which was significantly downregulated in inflamed mucosa of patients with ulcerative colitis, as one of inflammatory signature genes.[160] In addition, the genes associated with fatty acid synthesis such as fatty acid synthase (*Fasn*) and sterol regulatory element binding transcription factor 1(*Srebp1*) were significantly downregulated in the colons of patients with ulcerative colitis, which could be partially associated with the loss of liver X receptor (LXR).[161] Changes in the expression pattern of genes involved in fatty acid uptake, metabolism and synthesis may be partially a consequence of inflammation, but also seem to contribute substantially to IBD pathophysiology.[161] Downregulation of fatty acid metabolism was also shown in other inflammatory disorders and was linked to a relative state of malnutrition.[176]

(6) Other genes.

Not surprisingly, many other genes which have not been discussed here were upregulated or downregulated in the mucosa of IBD patients or animal models, in comparison to controls. [158-163, 128,130] Some of these genes might be involved in antigen presentation (e.g., genes encoding MHC I and MHC II), synthesis of immunoglobulin, cell adhesion, and tissue extravasation (e.g., genes encoding matrix metalloproteases, MMPs). For example, the MHC II genes associated with antigen-presentation were upregulated in colitis of TCR$\alpha^{-/-}$ mice, indicating the elevated antigen presentation activities in colitis.[130] Matrix metalloproteinases (MMPs) are responsible for the turnover and degradation of extracellular matrix and multiple MMPs (e.g., *MMP-1, MMP-3, MMP-7, MMP-9, MMP-13)* were significantly upregulated in colon of IBD patients, in comparison to control.[177-179]

Collectively, the general trend for IBD mucosal gene expression appears to be upregulation of genes associated with pro-inflammation, anti-inflammation, and cell growth/apoptosis along with the downregulation of genes associated with detoxification and fatty acid metabolism. These changes likely reflect the activation and regulation of immune responses, alteration of mucosal barrier functions, and metabolic activity, all of which are consistent with the major parameters believed to be associated with the development of IBD: aberrant immune responses, mucosal permeability, and genetic predisposition. Further more, this is consistent with the "multi-hit hypothesis" that the onset of IBD in immunocompetent hosts requires at least two triggers such as the alteration of mucosal barrier function and dysregulation of immune function.

Two animal models of intestinal inflammation used in this dissertation

In this dissertation, two animal models were used to study changes in gene expression associated with a predisposition to develop colitis (i.e., *H. bilis* –induced intestinal inflammation and *Brachyspira hyodysenteriae* –induced colitis). Those two models are novel and different from previous classical IBD animal models.

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***Helicobacter bilis* colonization in gnotobiotic C3H mice**

Helicobacter bilis is a microaerophilic, gram-negative spiral-shaped bacterium originally isolated from the livers and intestines of aged, inbred mice.[180] It is a member of an expanding and genetically diverse group of enterohepatic *Helicobacter spp* including *H. hepaticus*, *H. rodentium*, *H. muridarum*.[181] In addition to mice, *H. bilis* has been isolated from dogs, gerbils, rats, cats, and humans.[182,183] *Helicobacter bilis* and other enterohepatic *Helicobacter spp* are widely spread among research and commercial rodent colonies and might significantly confound interpretations of some research.[184,185]Additionally, experimental infection of a variety of immune competent and genetically-engineered (i.e., transgenic) mouse strains with *Helicobacter spp* have been used to develop animal models of gastrointestinal diseases as well as idiopathic human disease syndromes such as inflammatory bowel disease.[185]

Helicobacter bilis can either induce or accelerate the development of inflammatory bowel disease in immunodeficient rodents though *H. bilis* infection in immune competent

mice often results in subclinical disease.[61,62, 186-189] *SCID* mice inoculated either intraperitoneally or orally with *H. bilis* develop proliferative typhlocolitis.[186,187] Co-infection of mice with *H. bilis* and *H. rodentium* has been associated with a outbreak of colitis in a *SCID* mouse colony.[190] *Helicobacter bilis* alone or with *H. hepaticus* has been shown to accelerate the development of colitis in Mdr1a$^{-/-}$ mice.[61,62] In addition, *H. bilis* colonization induced moderate to severe colitis in IL-10$^{-/-}$ mice, TCR α $^{-/-}$ mice, and athymic rats.[188,189] The increased severity of colitis in IL-10$^{-/-}$ mice colonized with *H. bilis* correlates with the elevated expression of MHC class II and pro-inflammatory cytokines including IL-1α, IL-1β, and IL-1 receptor α in colon.[188] Collectively, these studies strongly support a hypothesis that *H. bilis* renders the immunodeficient host more susceptible to the development of colitis, though T cells might not be strictly essential in this process.

Emerging evidence suggests that subclinical *Helicobacter* infection may alter host responses to other experimental stimuli such as gut flora in unanticipated ways.[185] Consistently, the recent studies in our laboratory showed that, *H. bilis* colonization of immunocompetent C3H/HeN gnotobiotic mice triggered persistent antibody and cytokine responses to commensal bacteria in the absence of overt inflammation, indicating a dysregulation of gut homeostasis.[191-193] Since immune responses to commensal bacteria play a key role in IBD etiology,[1] it is hypothesized that immune responses to commensal bacteria induced by *H. bilis* colonization might initiate or perpetuate the development of IBD. Indeed, the data presented in Chapter 3 will show that *H. bilis* colonization increased host susceptibility to a subsequent colitic insult (e.g., low dose DSS), thus, *H. bilis* colonization in C3H/HeN gnotobiotic mice providing a novel IBD susceptibility model.

C3H/HeN:Tac gnotobiotic mice are colonized by only eight commensal bacteria in the gut known as Altered Schaedler's Flora (ASF) which includes ASF356, *Clostridium cluster XIV;* ASF360, *Lactobacillus acidophilus;* ASF361, *Lactobacillus murinus;*. ASF457, *Mucispirillum schaedleri;* ASF492, *Eubacterium plexicaudatum;* ASF500, *low-G+C content-positive bacteria;* ASF502, *Clostridium cluster XIV*; and ASF519, *Bacteroides distasonis*.[191] One of the major advantages of this model is that immune response to each commensal bacterium can be studied individually due to the nature of defined-flora.

In contrast to germ-free mice, these gnotobiotic C3H mice have normal anatomical and physiological functions. Also in contras to many immunodeficient IBD models, these mice are immunocompetent. In conclusion, *Helicobacter bilis* colonization of C3H/HeN:Tac gnotobiotic mice is a unique and practical animal model to chronologically study the induction of host susceptible factors associated with mucosal inflammation.

The studies related to the pathogenesis or virulence factors of *H. bilis* might greatly contribute to a better understanding of this IBD models. The biochemical characterization of *H.bilis* showed that *H. bilis* is urease, catalase and oxidase positive and can reduce nitrate to nitrite.[180] Several enterohepatic *Helicobacter* species including *H. bilis* and *H. hepaticus* contain cytolethal distending toxin (CDT), which that has the potential to induce distention in cells and to arrest cell division in the G2/M phase of cell cycle.[194] Cytolethal distending toxin has also been shown to play a key immunomodulatory role that facilitates persistent colonization of *H. hepaticus* and that, in IL-10$^{-/-}$mice, this alteration of the host immune response results in the development of colitis .[195] It is possible that CDT of *H. bilis* has similar functions to that of *H. hepaticus*.

***Brachyspira hyodysenteriae* –induced colitis**

Brachyspira hyodysenteriae, previously referred to as *Treponema hyodysenteriae* or *Serpulina hyodysenteriae,* is a gram-negative, anaerobic spirochete and is the causative agent of swine dysentery, a mucohemorrhagic diarrheal disease characterized by severe inflammation of cecum and colon.[196] Histological lesions in pigs include crypt elongation, superficial epithelial erosion, submucosal edema, inflammatory cell infiltration and mucosal hyperplasia etc.[197] These characteristics are similar to that of human ulcerative colitis. *Brachyspira hyodysenteriae* infection has been used as a model to study infectious or bacterial-induced colitis.[197-199]

In addition to pigs, several other species of animals including guinea pigs and chicks develop intestinal lesions after *B. hyodysenteriae* infection and have been utilized as alternative models.[200, 201] However, mice have been the most commonly used and well-documented laboratory animal model used to study the pathogenesis of swine dysentery.[202]

In contrast to pigs, the lesions induced by *B. hyodysenteriae* in mice model are less severe and limited to the ceca.

Intestinal inflammation induced by *B. hyodysenteriae* is characterized by the massive infiltration of inflammatory cells, particularly neutrophils. It has been shown that neutrophil depletion by anti-Ly6G treatment significantly reduced the intestinal lesions induced by *B. hyodysenteriae* infection.[202] In addition, anti-CD18 treatment also inhibited neutrophil infiltration and reduced intestinal lesions.[202] This study demonstrated the importance of neutrophil infiltration in the development of acute colitis induced by *B. hyodysenteriae* infection. *Brachyspira hyodysenteriae* infection also induced the temporal upregulation of pro-inflammatory cytokines (e.g., IL-1, IL-6, TNFα and MIP2) in murine cecal mucosa (unpublished data).

The exact pathogenesis of *B. hyodysenteriae*-induced colitis is not completely understood. However, several virulence factors including hemolysin and lipopolysaccharide (LPS) have been suggested. [203, 204] It has been shown that the hemolysin of *B. hyodysenteriae* induces similar early morphologic changes in mice ceca as observed following infection with *B. hyodysenteriae*.[203] On the other hand, endotoxin-like preparations such as butanol/water extract of *B. hyodysenteriae* can induce IL-1 and TNF production by murine peritoneal exudate cells (PECs), indicating that spirochetal components have pathological characteristics and potentially contribute to intestinal inflammation.[204]

Convincing evidence shows that the presence of gut flora is indispensible for the lesion development induced by *B. hyodysenteriae* in both pigs and mice.[205-208] Germ-free pigs or germ-free mice infected with *B. hyodysenteriae* alone did not develop intestinal lesions or clinical disease even though *B. hyodysenteriae* colonized the colon and invaded the cecal mucosa.[205-208] These results demonstrated that the presence of other commensal bacteria such as *Bacteroides vulgatus* is required for the development of colitis following infection with *B. hyodysenteriae*. Antibiotics have been widely used to treat swine dysentery induced by *B. hyodysenteriae* infection, generating concern for the development of antibiotic resistance bacteria.[209,210] The use of drugs that would regulate host inflammatory responses as opposed to antibiotic therapy including plant extracts such as hypoxoside may represent a novel treatment option for diseases such as those caused by *B. hyodysenteriae*.

In this dissertation, we first studied the mucosal gene expression profiles at 15, 30 and 45 days following the colonization of *Helicobacter bilis* in gnotobiotic immunocompetent mice. This study showed that *H. bilis* colonization could significantly and persistently downregulate the genes associated with detoxification processes, which is consistent with other microarray studies[128, 130, 178] and might predispose the host to the development of colitis. Secondly, we demonstrated that *H. bilis* colonization can exacerbate colitis induced by dextran sodium sulfate treatment. The effects of *H. bilis* might be associated with both downregulation of detoxification-associated genes and upregulation of genes involved in the activtation /recruitment of macrophage and T cells. Lastly, we switched to another other IBD model using *Brachyspira hyodysenteriae* - induced colitis. We were able to determine that hypoxoside, an anti-inflammatory drug extracted from an African traditional medicine *Hypoxis hemerocallidea* could ameliorate the colitis induced by *B. hyodysenteriae* infection. More importantly, the anti-inflammatory effects of hypoxoside appeared to be associated with the reduction of neutrophil infiltration, downregulation of genes involved in the NF-κB signaling pathways and the detoxification process resulting in decreased epithelial cell proliferation, but not anti-microbial activity. In conclusion, the studies in this dissertation suggest differential mucosal gene expression induced by different agents such as with a mild bacterial pathogen, *Helicobacter bilis* or use of an anti-inflammatory drug, hypoxoside could alter the mucosal epithelial barrier functions and/or immune regulation of lamina propria, and then modulate the process of murine colitis development.

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CHAPTER 2: MUCOSAL GENE EXPRESSION PROFILES FOLLOWING THE COLONIZATION OF IMMUNOCOMPETENT GNOTOBIOTIC C3H MICE WITH *Helicobacter bilis:* *A PRELUDE TO COLITIS*

A paper to be submitted to the journal *Inflammatory Bowel Diseases*

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ABSTRACT

Mechanisms associated with the development of inflammatory bowel diseases include aberrant immune responses to the resident flora, changes in mucosal barrier function, and genetic predisposition of the host. It has been shown that colonization of gnotobiotic mice with *Helicobacter bilis* triggers host immune responses to the commensal flora. The mechanisms by which *H. bilis* colonization affects mucosal homeostasis and the development of immune responses to the flora have not been determined. Using microarray analysis, changes in mucosal gene expression contributing to an increased susceptibility to colitis were evaluated following *H. bilis* colonization. Differential gene expression ($q \leq 0.05$, fold change ≥ 2) within the cecal mucosa was assessed at 15, 30, or 45 days following *H. bilis* colonization using Affymetrix Genechips. The expression of selective genes was confirmed by quantitative RT-PCR. *H. bilis* colonization induced marked upregulation of genes associated with protein metabolism, immune responses, and downregulation of genes associated with fatty acid metabolism and detoxification which peaked at 15 days postinfection. A set of genes associated with glycoprotein synthesis and detoxification including *Fut2, B3galt5*, *Ceacam12, Cyp4b1,* and *Ugt8a* were uniquely identified and found

to be similarly expressed following the induction of colitis by dextran sodium sulfate or *Brachyspira hyodysenteriae*.In addition to the immune response-associated genes (e.g., interferon-inducible genes), genes associated with mucosal homeostasis, such as glycoprotein synthesis and detoxification, were shown to be differentially expressed and these changes correlated with the observed increased susceptibility to colitis induced by *H.bilis* colonization of gnotobiotic mice.

Key Words: *Helicobacter bilis*, gene expression, mucosa, susceptibility, colitis

INTRODUCTION

Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are characterized by chronic, relapsing inflammation of the gastrointestinal tract. Although the exact etiology of the inflammatory disorders remains unknown, it is widely hypothesized that dysregulated innate and/or adaptive immune responses to antigens derived from the intestinal microflora contribute to the onset and/or severity of IBD.[1-3]

Helicobacter bilis is a microaerophilic, gram-negative spiral-shaped bacterium and causes mild, subclinical inflammation in immunocompetent hosts.[4] Several studies have linked *H. bilis* to the development of or acceleration of proliferative typhlocolitis in immunocompromised rodents such as SCID, Mdr1a$^{-/-}$, IL-10$^{-/-}$ and TCR$\alpha^{-/-}$mice, as well as athymic nude rats.[5-9] Using immunocompetent C3H/HeN gnotobiotic mice, which harbor the eight members of the altered Schaedler's flora (ASF), previous studies have shown that *H. bilis* colonization did not induce demonstrable inflammation yet triggered persistent antibody and cytokine responses to antigens derived from the resident flora, which were not observed in control mice.[10-12] These results indicate that introduction of a novel bacterial species such as *H. bilis* into the gastrointestinal microbiota alters mucosal homeostasis which may increase the host's susceptibility to the development of colitis. In contrast to many other IBD models using mice with various degrees of immunodeficiency and/or undefined microflora, immunocompetent gnotobiotic mice colonized with *H. bilis* provides a unique and practical model to study the roles of host immune responses to specific commensal bacteria in the development of IBD. However, the molecular mechanisms affecting the alteration of immune responses induced by *H. bilis* colonization remain unknown.

The advance of microarray technology has greatly expanded our ability to systematically monitor the effect of pathogens on the host gene expressions.[13] In this study, Affymetrix Genechips were employed to study global and temporal gene expression profiles of cecal mucosa following the colonization of gnotobiotic mice with *H. bilis.* The differentially expressed genes were functionally classified and linked to host susceptibility to colitis. In addition to the immune response-associated genes, some genes associated with gut physiological homeostasis such as glycoprotein synthesis and detoxification were found to be differentially expressed and likely contributed to the increased susceptibility to colitis induced following *H. bilis* colonization. To assess whether or not the observed changes in mucosal gene expression were common to other colitic insults, two other models of colitis (dextran sodium sulfate and *Brachyspira hyodysenteriae*) were used to evaluate changes in gene expression. The results of these studies identified a set of genes with similar changes in their pattern of gene expression as previously reported for other IBD animal models. Using an immunocompetent gnotobiotic mouse model, this study provides preliminary evidence and insights as to the types of factors or changes induced within the intestinal mucosa that might predispose the host to the development of colitis.

MATERIAL AND METHODS

Animals and Treatments. Eight to nine week old, gnotobiotic C3H/HeN: TAC mice colonized with the ASF were obtained from Taconic Farms (Germantown, NY) and gnotobiotically maintained at Iowa State University. Members of the ASF include ASF356, *Clostridium cluster XIV;* ASF360, *Lactobacillus acidophilus;* ASF361, *Lactobacillus murinus;* ASF457, *Mucispirillum schaedleri;* ASF492, *Eubacterium plexicaudatum;* ASF500, *low-G+C content-positive bacteria;* ASF502, *Clostridium cluster XIV*; and ASF519, *Bacteroides distasonis*. All animal-related procedures were approved by the Animal Care and Use Committee at Iowa State University.

Helicobacter bilis was cultivated and harvested as described.[10] Mice were orally colonized once daily over three consecutive days with approximately 10^8 CFU of *H. bilis*.

Experimental colitis was induced by dextran sodium sulfate (DSS) (MP Biomedicals, Solon, OH), which was dissolved in drinking water to a concentration of 2% (weight/volume)

or oral infection with 10^8 CFU *Brachyspira hyodysenteriae* [10]. The spirochetal infection was confirmed by demonstration of β-hemolytic spirochetes recovered from colonic contents. Five days after DSS treatment or seven days after *B. hyodysenteriae* infection, cecal tissues were collected and total RNA were isolated using the RNeasy Mini Kit and RNase-Free DNase according to the manufacture's protocol (Qiagen, Valencia, CA).

Experimental design. Mice were randomly assigned into three pairs for each of four separate treatment groups (Control, *H. bilis* 15 DPI, *H. bilis* 30 DPI, and *H. bilis* 45 DPI). Two mice from each treatment group were assigned to a separate Genechip. Mice were inoculated with *H. bilis* at 15, 30, or 45 days prior to necropsy. *H. bilis* colonization was confirmed by both PCR [10] and bacteriological culture. At necropsy, samples of cecal tissues obtained from two mice within the same treatment were pooled in order to reduce the potential variability associated with evaluating individual tissue samples. Total RNA was isolated from cecal tissue samples as described above.

Histology. Microscopic lesion scores were evaluated as previously described.[10] Briefly, tissues were formalin-fixed, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E). Stained cecal sections were scored by a pathologist (Dr. J. M. Hostetter, Department of Veterinary Pathology, Iowa State University) in a blinded fashion. Histological scores were based on the severity of mucosal epithelial damage, degree of lamina propria cellular infiltrate, and architectural distortion.[10]

Affymetrix oligonucleotide array analysis. Total RNA samples extracted from cecal tissues were submitted to the Iowa State University Genechip Facility (Ames, IA). Labeling of samples, generation of cRNA, hybridization, staining, and scanning were performed according to protocols in the Affymetrix Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). Affymetrix Mouse genome 430 2.0 arrays were used in this experiment.

Fluorescence intensity values (.CEL files) generated from hybridized and labeled genechips were analyzed using R software (http://www.r-project.org) and a Bioconductor analysis package (http://www.bioconductor.org). Data were normalized using Robust Multi-array Average (RMA).[14] False discovery rate (*q* value) of normalized data was computed using Storey and Tibshirani's default method.[15] Differentially expressed genes were statistically filtered using a q value ≤ 0.05 and a estimated fold change relative to control

values ≥ 2 or ≤-2. The differentially expressed genes were clustered using both hierarchical cluster via Pearson correlation and K-means methods. Assignments of filtered genes to functional categories were performed by using Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics resource 2007 (National Institutes of Health, Bethesda)[16] and/or literature-based classification schemes. Fisher exact tests were performed to calculate *P* values and determine which particular annotation categories were over-represented.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR). Total RNA samples from mouse ceca were treated with Turbo DNA-free DNase (Ambion, Austin, TX) to remove genomic DNA contamination and were then reverse transcribed into cDNA by SuperScript™ III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). The cDNA converted from 20 ng RNA was amplified using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen). The PCR conditions used for these studies were established as follows: 95 ℃ for 10 minutes, and then followed by 40 cycles of amplification (95 ℃ for 15 seconds, 60 ℃ for 60 seconds). The relative mRNA quantities were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and control samples. The quantitative PCR was performed using a Rotor-Gene 3000 (Corbett Research, Mortlake**,** Australia). Standard curves for the selected genes and GAPDH were made using serial dilutions of cDNA. The correct sizes of the amplified PCR products were confirmed by gel electrophoresis. The primer sequences used in these studies are available on request.

Statistics. The data are expressed as mean ± SEM. The group comparisons were made using the robust Kruskal–Wallis non-parametric analysis of variance (ANOVA). If comparisons were statistically significant ($P<0.05$), then an analysis of the pairwise means were compared using a two-sided Wilcoxon rank-sum test.

RESULTS

Histological lesions. It has been shown that colonization of immunocompetent C3H gnotobiotic mice with *Helicobacter bilis* triggers persistent host immune responses to the resident commensal bacteria without the induction of overt macroscopic or microscopic inflammation [10, 11]. In these studies, *H. bilis* colonization of gnotobiotic mice resulted in

subtle histological changes in cecal and colonic mucosa characterized by small increases in the density of lymphocytes and plasma cells within the lamina propria, and small increases in mucosal height. While the histological lesion scores at 15 DPI were higher than those at 30 and 45 DPI, there was no statistically significant difference observed by light microscopy (Fig. 1). As previously demonstrated,[10, 11] *H. bilis* colonization of gnotobiotic mice induced antigen-specific antibody responses to resident ASF that was observed at 15, 30, and 45 DPI (data not shown). Collectively, these data indicated that there were slight morphological changes within the colonic mucosa following *H. bilis* colonization that did not directly correlate with the development of host immune responses to the resident flora or the increased sensitivity of these mice to a subsequent colitic insult such as of DSS (Chapter 3). To extend these observations, changes in the transcriptional profile of the cecal mucosa was evaluated to elucidate the effect of *H. bilis* colonization on mucosal homeostasis.

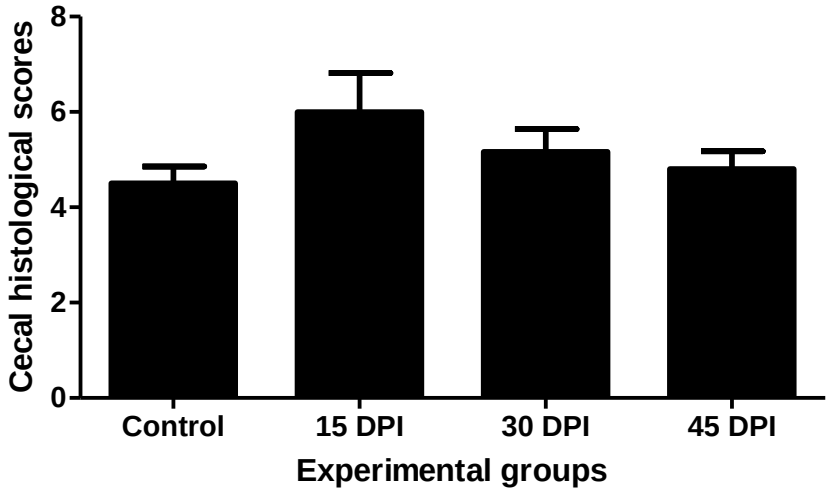

Figure 1. Evaluation of cecal histological lesions of gnotobiotic mice following the colonization of *Helicobacter bilis.* Cecal tissues (0, 15, 30, 45 DPI) were stained with hematoxylin and eosin (H&E) and evaluated by a pathologist in a blinded fashion. Histological scores were determined as outlined in "Materials and Methods". Bars represent the mean ± SEM; n=4-6 mice/group. There was no statistical difference between any of the groups.

Mucosal gene expression after *H. bilis* colonization.

Overall gene expression patterns and functional annotation analysis

In the absence of overt mucosal inflammation, it was hypothesized that *H. bilis* colonization would induce changes in the pattern of gene expression that would alter mucosal homeostasis. To evaluate the effect of *H. bilis* colonization on mucosal homeostasis, microarray analysis was performed at three separate time points following colonization.

Scatterplots of overall gene expression profiles across experimental groups showed variable degrees of correlation between samples obtained from control mice versus those colonized with *H. bilis*. As indicated by the number of dots above or below the diagonal line, there was a marked discorrelation between the pattern of gene expression at 15 DPI and the controls (Fig. 2A). Similar levels of moderate discorrelation of gene expression levels existed between samples from mice infected for 30 or 45 DPI versus control mice (Fig. 2B and data not shown). However, there was high correlation between samples collected at 30 and 45 DPI (Fig. 2C). These data indicate that the overall gene expression profiles at 15 DPI differ from those of 30/45 DPI which may represent acute versus chronic changes in mucosal gene expression.

Using q value ≤ 0.05 and an estimated fold change relative to control values ≥ 2 or ≤ -2, 115 differentially expressed genes were identified following *H. bilis* colonization, which could be separated into three patterns of gene expression by K-means cluster analysis (Fig. 3). Pattern 1 revealed a set of genes whose expression levels were upregulated at 15 DPI and returned to near background levels by 30 DPI (Fig. 3A). Pattern 2 defined a group of genes whose expression levels were upregulated at 15 DPI and remained elevated through 45 DPI (Fig. 3B). Pattern 3 included genes that were maximally down-regulated by 15 DPI and remained down-regulated through 45 DPI (Fig. 3C).

Hierarchical clusters showed that, in comparison to samples from control mice, the greatest numbers of genes were differentially expressed at 15 DPI. In addition, there were few differentially expressed genes when samples from mice colonized for 30 and 45 days were compared (Fig. 4). This data was consistent with the correlation of overall gene expression profiles after *H. bilis* colonization as depicted by the scatterplot analysis in Figure 2 (i.e., tighter clustering along the diagonal line).

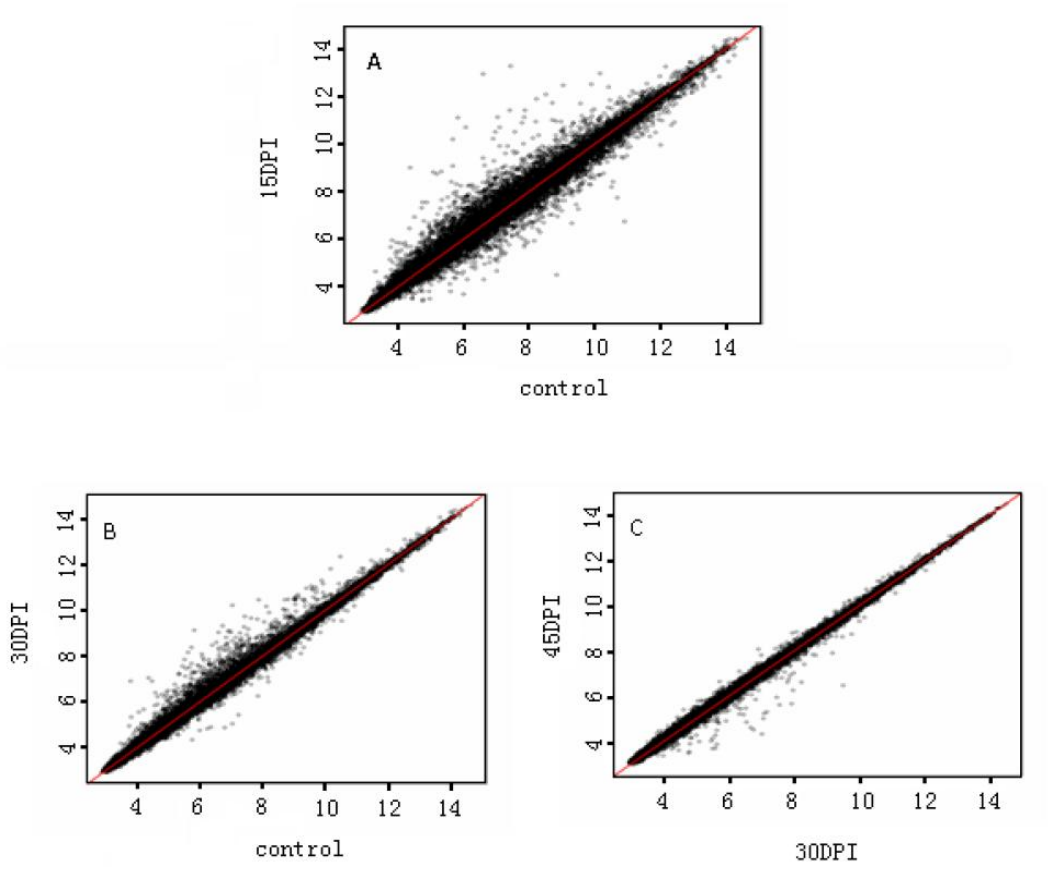

Figure 2. Scatterplot analysis depicting comparative changes in mRNA expression over time following the colonization of gnotobiotic mice with *Helicobacter bilis*. Changes in gene expression over time were expressed as the overall signal intensity of all genes in the cecal mucosa of gnotobiotic mice at 0, 15, 30 or 45 days after *H.bilis* colonization. Dots above the diagonal line represent upregulated genes while dots below the diagonal line represent down-regulated genes. (A) Comparison between samples collected at 15 DPI vs. control; (B) 30 DPI vs. control; (C) 45 DPI vs. 30 DPI. n=6 mice/group.

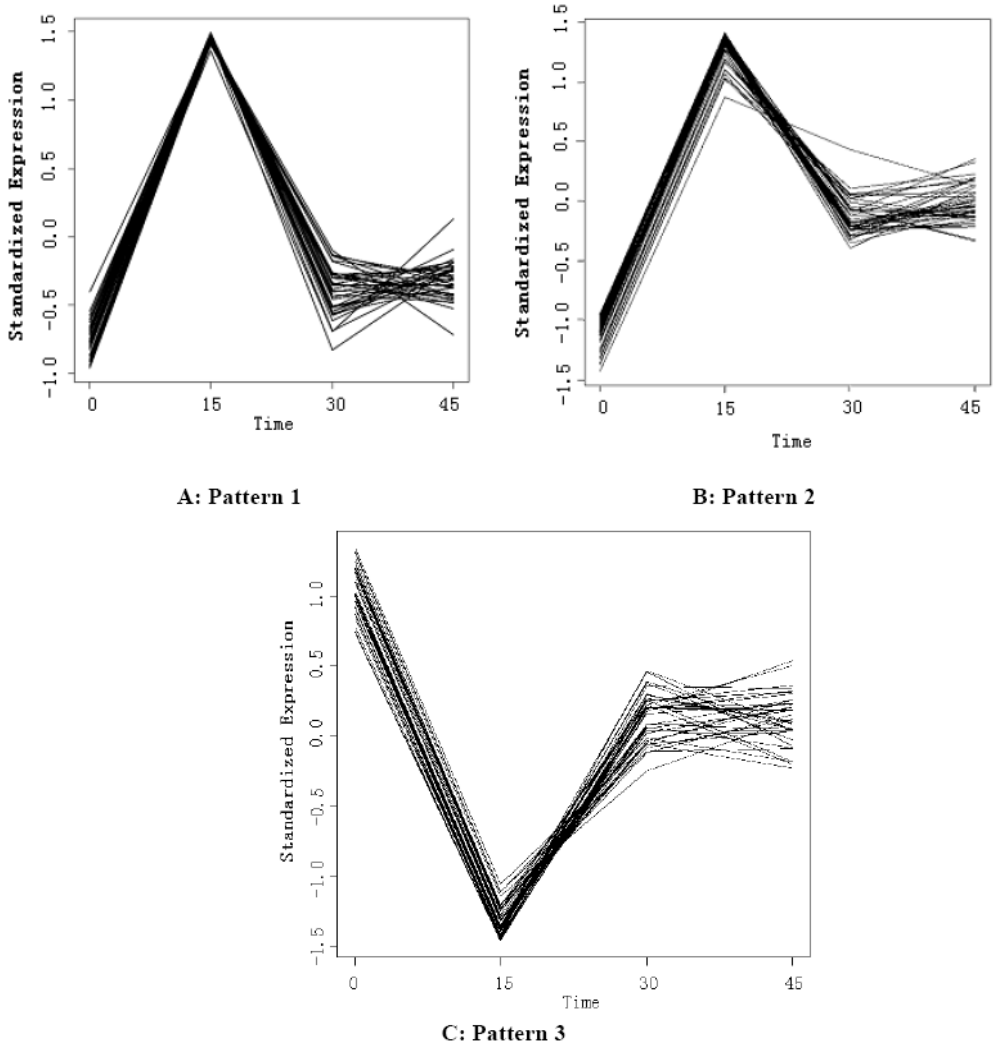

Figure 3. Patterns of differentially expressed genes as determined by K-means cluster analysis following the colonization of gnotobiotic mice with *Helicobacter bilis*. Differential mucosal gene expression was defined using a q value ≤ 0.05 and fold change relative to control ≥ 2 or $\leq$-2. Three separate temporal patterns (1, 2, and 3) of gene expression were detected. The average expression levels of each gene across all time points were standardized to zero. (A) Pattern 1 contained 39 genes (B) Pattern 2 contained 41 genes (C) Pattern 3 contained 35 genes. n=6 mice/group. 0: Control, 15: 15 DPI, 30: 30 DPI, 45: 45 DPI.

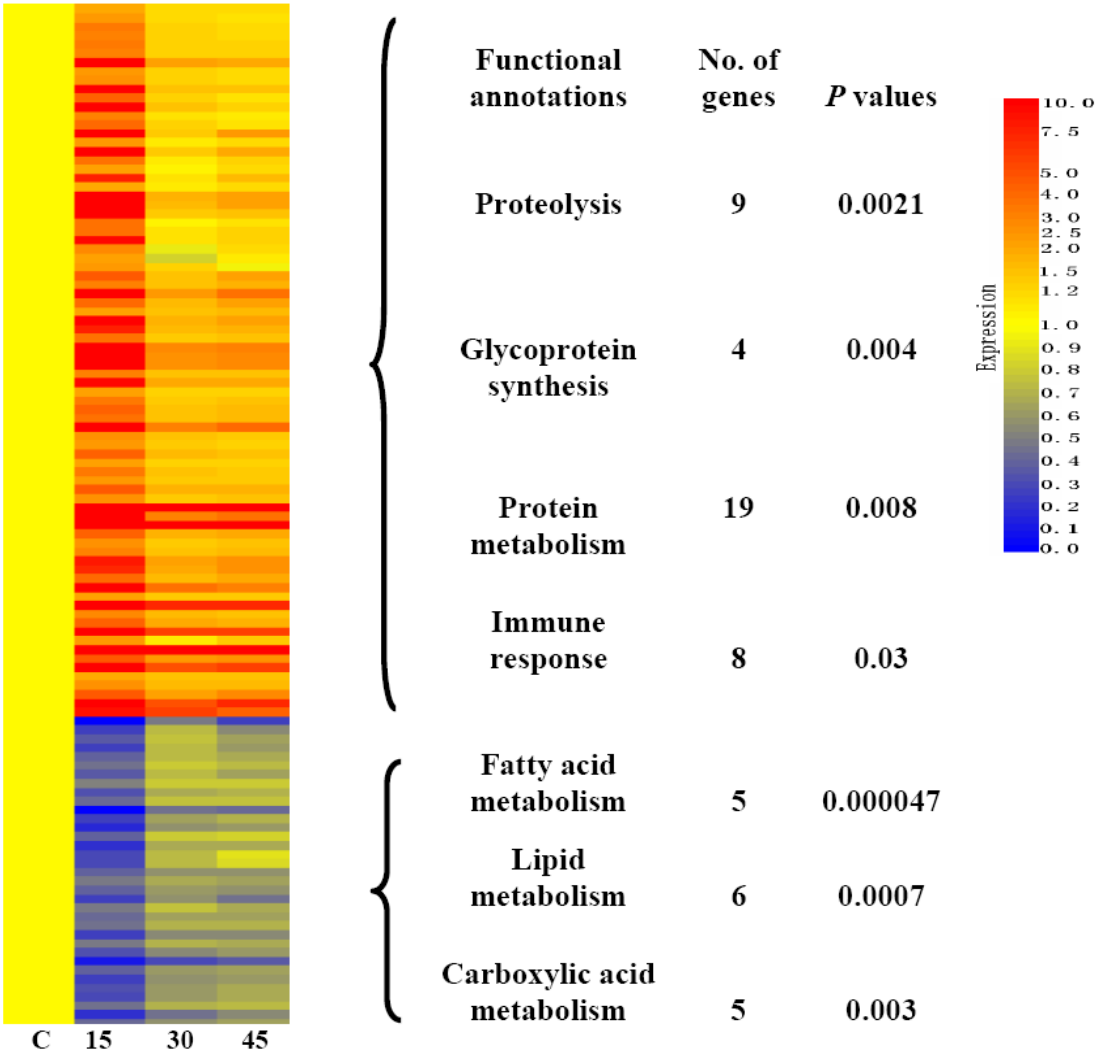

Figure 4. Hierarchical cluster analysis and functional annotation of differentially expressed genes. Genes were clustered using Pearson correlation. Functional annotation of the differentially expressed genes (q value ≤ 0.05 and fold change relative to control samples of ≥ 2 or $\leq$-2) for samples obtained from gnotobiotic mice at 15, 30 or 45 days following colonization with *Helicobacter bilis* are depicted. Orange to red colors represent upregulated genes over control, and gray to blue colors represent downregulated genes over control. The color bar and relative fold change it represents is denoted on the right. The gene lists were ontologically grouped using the DAVID bioinformatics resource 2007. *P* values were calculated using Fisher exact probability test and were used to determine the over-represented functional annotations. C: Control; 15: 15 DPI; 30: 30 DPI; 45: 45 DPI.

The lists of differentially expressed gene were analyzed using the DAVID bioinformatics resource 2007 and several over-represented gene categories were identified. According to the *P* values and literature-based classification schemes, the most over-represented ontological categories of upregulated genes were associated with protein metabolism (e.g., proteolysis and glycoprotein synthesis) and immune responses, while the most over-represented downregulated genes were associated with fatty acid metabolism and detoxification processes (Fig.4 , Table 2).

Functional annotation analysis of K-means clusters showed that the expression profiles of genes associated with proteolysis and immune responses (e.g., *Cd14*) were in pattern 1 (Table 1). Genes associated with glycoprotein synthesis and immune responses (e.g., IFN-inducible genes) were in pattern 2 (Table 1) while downregulated genes were associated with pattern 3 (Table 2). These results showed that expression levels of genes associated with glycoprotein synthesis and fatty acid metabolism or detoxification processes tended to stay upregulated or downregulated through 45 DPI, respectively. While elevated at 15 DPI, expression levels of some genes associated with proteolysis and innate immune responses returned to control levels by 30 DPI.

Analysis of upregulated genes

In addition to initiating immune responses to counter the impact of bacterial colonization, the host will often adjust metabolically and physiologically in an effort to maintain tissue homeostasis. An influx of inflammatory cells and elevated levels of serine proteases have been found in the intestine during inflammation, infection, and stress.[17] In this study, we detected upregulation of genes associated with protein metabolism along with changes in immune response genes following colonization with *H. bilis* (Table 1). Following colonization by *H. bilis*, we observed marked upregulation of granzyme genes including an approximately 30-fold increase in *Gzma*, *Gzmb* at 15 DPI (Table 1). In addition, genes encoding mast cell proteases *Mcpt1*, *Mcpt2*, *Mcpt9*, and *Cpa3* and mast cell surface receptors *Fcer1a* were upregulated 8-55 fold at 15 DPI (Table 1). The expression profile of these genes fell into pattern 1 (Table 1) as their level of expression returned to control levels by 30 DPI. The change patterns of proteolysis-associated genes may correlate with the level of inflammation.

Table 1. Selected upregulated genes in cecal mucosa of gnotobiotic mice following the colonization with *Helicobacter bilis*.

Genebank ID	Gene description[a]	Fold change			Functional annotations[b]	Pattern
		15DPI	30DPI	45DPI		
NM_010370	Gzma: granzyme A	29.9	1.6	1.5	Proteolysis /Immune response	1
NM_013542	Gzmb: granzyme B	34.3	1.8	2.2	Proteolysis	1
NM_008570	Mcpt1: mast cell protease 1	54.8	2.9	3.3	Proteolysis	1
NM_008571	Mcpt2: mast cell protease 2	35.6	2.6	2.8	Proteolysis	1
AY007568	Mcpt9: mast cell protease 9	12.4	1.5	1.3	Proteolysis	1
NM_007753	Cpa3: carboxypeptidase A3, mast cell	8.1	1.6	1.9	Proteolysis	1
AF114266	Tgm2: transglutaminase 2, C polypeptide	2.3	1.0	1.2	Proteolysis	1
NM_009425	Tnfsf10: tumor necrosis factor (ligand) superfamily , member 10	2.5	1.3	1.2	Immune response	1
NM_009841	Cd14: CD14 antigen	2.3	1.2	1.1	Immune response	1
NM_011558	Tcrg: T-cell receptor gamma chain	4.6	1.3	1.1	Immune response	1
NM_010184	Fcer1a: Fc receptor, IgE, high affinity I, alpha polypeptide	11.3	1.3	1.6	Immune response	1
NM_018876	Fut2: fucosyltransferase 2	9.3	2.0	2.0	Protein amino acid glycosylation	2
AI481328	Mgat2: mannoside acetyl-glucosaminyltransferase 2	2.6	1.4	1.5	Protein amino acid glycosylation	2
BB430637	B3galt5: UDP-Gal: betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 5	92.2	6.1	6.0	Protein amino acid glycosylation	2
BF578266	B3gnt7: UDP-GlcNAc:betaGal beta -1,3-N-acetyl-glucosaminyltransferase 7	41.6	3.1	4.3	Protein amino acid glycosylation	2
NM_026087	Ceacam12: CEA-related cell adhesion molecule 12	101.9	14.1	17.2	Unknown	2
NM_010259	Gbp1: guanylate nucleotide binding protein 1	4.4	1.5	1.7	Immune response	2
BE197524	Gbp2: guanylate nucleotide binding protein 2	3.7	1.5	1.7	Immune response	2
BM245961	LOC667373: similar to interferon-induced protein with tetratricopeptide repeats 1	16.6	5.1	7.3	Immune response	2

[a] Genes were selected by using q value ≤ 0.05, fold change relative to control ≥ 2.
[b] Functional annotations were identified by DAVID bioinformatics resource 2007.

The upregulation of genes associated with the immune response included *Cd14* and multiple IFN-inducible genes (Tables 1 and 2). IFN-inducible genes included P47 GTPase (e.g., *Ifi47),* P65 GBP (e.g., *Gbp1*, *Gbp2*, and *Loc667373*), and Mx gene families and have been implicated in the immune response against bacteria.[18] Interestingly, the expression change of *Cd14* (returned to control levels, pattern 1) and IFN-inducible genes (remained elevated, pattern 2) belong to different gene expression patterns.

Changes in the fucosylation or glucosylation of epithelial cell surface molecules have been noted following bacterial colonization.[19, 20] Remodeling of cellular surface structures likely reflects an attempt by epithelial cells to adjust to changes in the bacterial flora. In this regard, four genes associated with glycoprotein synthesis (*Fut2, Mgat2, B3galt5, B3gnt7*) were significantly upregulated at all three time points. Two of those genes (*Fut2* and *B3galt5*) are involved in the synthesis of type 1 Lewis antigens.[21] In addition, *Ceacam12* gene was strongly upregulated 15 days after *H. bilis* colonization and stayed upregulated at 30 and 45 DPI. The fold changes over control for *Ceacam12* were >40 fold at 15 DPI, and >10 fold at 30 and 45 DPI (Table 1 and Fig. 5).

Analysis of downregulated genes

In addition to being precursors of inflammatory mediators, fatty acids exert nutritional and protective effects on enterocytes. Altered fatty acid metabolism has been previously reported in humans with inflammatory bowel diseases.[22-24] Of the differentially expressed genes with known or predicted functions, the category with the most downregulated genes included those associated with fatty acid metabolism (Table 2). Downregulation of some genes such as *Acsl1 that are* involved with fatty acid biosynthesis might revive an earlier hypothesis that energy deficiency is a significant factor in pathogenesis of colitis.[25, 26]

Using literature-based classification schemes, some of the downregulated genes were associated with detoxification and/or biotransformation processes (Table 2), which included drug transporters, phase I and phase II metabolic enzymes.[27-29] Downregulated genes associated with phase I metabolism enzymes included carboxylesterase (*Ces1*, *Ces3*), cytochrome P450 gene family (*Cyp4b1*, *Cyp4f1*), and epoxide hydrolase (*Ephx2*). Downregulated phase II metabolism enzymes included glucuronyl transferase (*Ugt8*) and glutathione transferase (*Gstt1*). Notably, *Cyp4b1* and *Ugt8a* genes were strongly

downregulated at 15 DPI with a high magnitude of fold changes over controls and remained downregulated at 30 and/or 45 DPI, in comparison to control (Table 2, Fig. 5).

Table 2. Selected downregulated genes in cecal mucosa of gnotobiotic mice following the colonization with *Helicobacter bilis*.

		Fold change				
Genebank ID	**Gene description[a]**	**15DPI**	**30DPI**	**45DPI**	**Functional annotations[b]**	**Pattern**
NM_008149	Gpam: glycerol-3-phosphate acyltransferase,mitochondrial	-3.8	-1.8	-1.7	Fatty acid metabolism	3 3
BI413218	Acsl1: acyl-CoA synthetase long-chain family member 1	-3.0	-1.3	-1.6	Fatty acid metabolism	3 3
NM_013703	Vldlr: very low density lipoprotein receptor	-4.2	-1.7	-2.2	Fatty acid metabolism	3
AB078618	Acot12:acyl-CoA thioesterase 12	-4.1	-1.4	-1.9	Fatty acid metabolism	3
NM_021456	Ces1: carboxylesterase 1	-3.0	-1.6	-1.5	Detoxification	3
AI315015	Ces3: carboxylesterase 3	-3.2	-1.5	-1.5	Detoxification / Fatty acid metabolism	3
NM_007823	Cyp4b1: cytochrome P450 , family 4, subfamily b, polypeptide 1	-65.6	-2.1	-4.2	Detoxification	3 3
NM_130882	Cyp4f13: cytochrome P450, family 4, subfamily f, polypeptide 13	-2.0	-1.3	-1.5	Detoxification	3 3
NM_007940	Ephx2: epoxide hydrolase 2, cytoplasmic	-4.1	-1.9	-1.9	Detoxification	3
BC012254	Gstt1: glutathione S-transferase, theta 1	-2.5	-1.8	-1.6	Detoxification	3
NM_011674	Ugt8a: UDP galactosyltransferase 8A	-79.5	-2.3	-2.3	Detoxification	3

[a] The genes were selected by using q value ≤ 0.05, fold change relative to control ≤ -2.

[b] Functional annotations were identified by either DAVID bioinformatics resource 2007 or literature-based classification schemes

Detoxification genes have been shown to be regulated by three separate nuclear receptors including pregnane X receptor (*Pxr*), constitutive androstane receptor (*Car* , also referred as *Nrli3*), and peroxisome proliferator-activated receptor alpha (*Ppara*).[30] The suppression of *Pxr* was linked to the downregulation of detoxification genes in IBD patients.[29] In this study, qRT-PCR results showed that *H. bilis* colonization induced the significant downregulation of *Car* gene, rather than *Pxr* and *Ppara* gene (Fig. 5 and data not shown).

Confirmation of Genechip data by qRT-PCR. To confirm the Genechip results, genes from different functional annotation groups were chosen for qRT-PCR analysis and included *Gzmb, Mcpt2, Fut2, B3galt5, Ceacam12, Cyp4b1, Ugt8a* and interferon-inducbile gene *Ifi47* (Supplemental data). Expression of all selected genes demonstrated similar trends of change by both qRT-PCR and microarray analysis at each of the specific time points tested (Fig. 5).

Analysis of gene expression patterns in other colitis models. In order to assess whether the changes in gene expression were unique to *H. bilis* colonization, changes in gene expression associated with two other models of colitis were evaluated. Dextran sodium sulfate (DSS) is a commonly used chemical to induce acute colitis in mice.[31] *Brachyspira hyodysenteriae* is the causative bacterium of swine dysentery and induces typhlocolitis in mice and pigs [32]. The expressions of a selected number of genes associated with mucosal inflammation (*Fut2, B3galt5, Ceacam12, Cyp4b1 and Ugt8a)* were evaluated following the onset of colitis induced by either DSS treatment or *B. hyodysenteriae* infection. Similar to the gene expression changes observed following the colonization with *H. bilis*, comparable changes in the gene expression of *Fut2*, *B3galt5, Ceacam12, Cyp4b1* and *Ugt8a* were noted within the cecal mucosa following DSS treatment or *B. hyodysenteriae* infection (Fig. 6A and B).

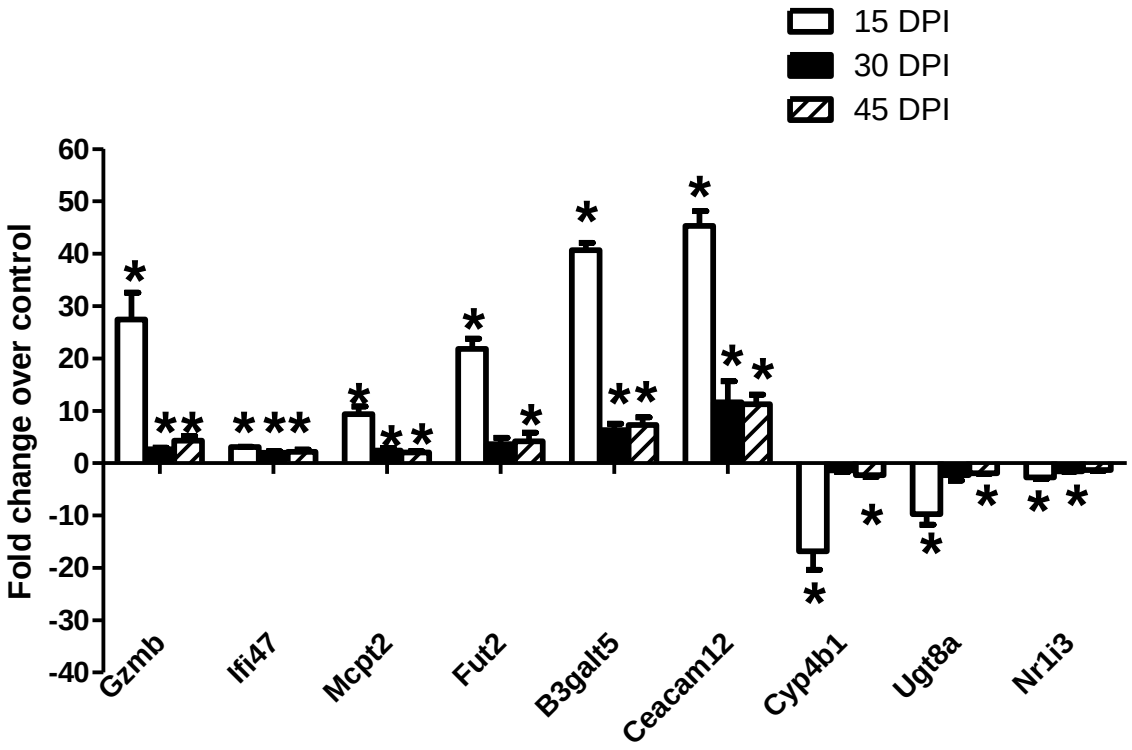

Figure 5. Analysis of mRNA expression via qRT-PCR in the ceca of gnotobiotic mice following the colonization of *Helicobacter bilis*. Cecal tissues were collected at 0, 15, 30, or 45 days from separate groups of mice after *H. bilis* colonization. Total RNA was isolated, converted into cDNA, and. amplified using gene-specific primers as described in "Material and Methods". Expression of each individual gene was initially normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the fold changes calculated as the relative difference to control samples. Each value represents mean ± SEM, n=6 mice. * $P<0.05$ vs. control. *Gzmb*: Granzyme B. *Ifi47*: Interferon gamma inducible protein 47. *Fut2*: Fucosyltransferase 2. *B3galt5*: UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 5. *Ceacam12*: CEA-related cell adhesion molecule 12.*Cyp4b1*: Cytochrome P450, family 4, subfamily b, polypeptide 1. *Ugt8a*: UDP galactosyltransferase 8A. *Nrli3*: Nuclear receptor subfamily 1, group I, member 3.

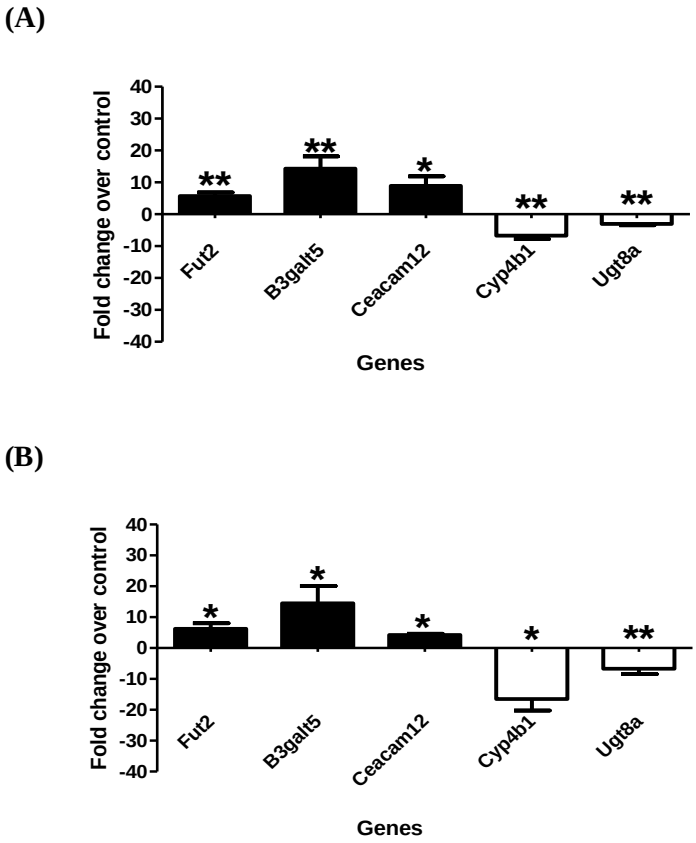

Figure 6. Comparative analysis of mRNA expression of novel colitis-associated genes in the cecal mucosa of gnotobiotic mice after dextran sodium sulfate (DSS) treatment or *Brachyspira hyodysenteriae* infection. Mice were treated with 2% DSS in drinking water or infected with *B. hyodysenteriae* as described in Material and Methods. Total RNA was isolated from cecal tissues and the relative mRNA expression of specific genes was assessed by qRT-PCR. (A) Fold change over control after DSS treatment (B) Fold change over control after *B. hyodysenteriae* infection. Individual gene expression values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and fold changes calculated as the relative difference to control samples. Each value represents mean ± SEM, n= 4-7 mice. * $P<0.05$ vs. Control, ** $P<0.01$ vs. Control. Filled bars represent the upregulated genes and open bars represent the downregulated genes. *Fut2*: Fucosyltransferase 2. *B3galt5*: UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 5. *Ceacam12*: CEA-related cell adhesion molecule 12.*Cyp4b1*: Cytochrome P450, family 4, subfamily b, polypeptide 1. *Ugt8a*: UDP galactosyltransferase 8A.

DISCUSSION

The current paradigm for the etiology of IBD includes the onset of inflammation induced by aberrant immune responses to commensal bacteria in genetically susceptible hosts [1-3]. Yet, the extreme complexity of resident microflora in conventional or specific pathogen free mice makes it difficult to dissect the roles of specific commensal bacteria to modulate intestinal inflammation.[11] However, the use of gnotobiotic mice would permit a more refined analysis of the impact individual bacterial species may exert on mucosal homeostasis and susceptibility to colitis. Several studies have linked *H. bilis* to the development or acceleration of proliferative typhlocolitis in immunocompromised rodents.[5-9] In this regard, recent studies from this laboratory have evaluated the impact of *H. bilis* colonization on the development of cellular and humoral immune response of C3H gnotobiotic mice to the individual members of the resident flora. [10, 11] These results indicated that introduction of novel bacterial provocateurs such as *H. bilis* into the gastrointestinal microbiota alters mucosal homeostasis and increases the host's susceptibility to the development of colitis. However, the molecular mechanism by which *H. bilis* colonization evokes the host response to commensal bacterial in mice remains unknown. In addition to the changes in the host's immune responsiveness to the resident flora, analysis of mucosal gene expression following colonization with *H. bilis* should provide insights with respect to changes in mucosal homeostasis that are predisposing towards rather than the direct underpinnings of acute or chronic inflammation.

Using microarray analysis, *H. bilis* colonization of gnotobiotic mice induced notable upregulation of genes involved in protein metabolism (e.g., proteolysis, glycoprotein synthesis) and immune responses while downregulating genes involved in fatty acid metabolism and detoxification. Many genes associated with mucosal physiology and homeostasis were identified. Similar changes in gene expression associated with intestinal physiology have been observed in germ-free mice following colonization with *Bacteroides thetaiotaomicron*.[20]

The magnitude of changes in gene expressions was greatest at 15 DPI suggesting that the mucosal tissue temporally regulated gene expression in an attempt to reestablish homeostasis

by 45 DPI. However, from qRT-PCR and K-means cluster analysis, the expression levels of some genes at 30 and 45 DPI, especially genes associated with glycoprotein synthesis and detoxification remained differentially expressed in comparison to control mice. In response to a subsequent colitic insult, the small but significant alterations of mucosal gene expression observed at 45 DPI would likely predispose the host to the development of or increase the susceptibility to chronic colitis. These observations would be consistent with a "multiple hit hypothesis" leading to the development of chronic colitis.[33] In this regard, preliminary data has shown that exposure to an otherwise innocuous dose of DSS induced severe colitis in *H. bilis*-colonized gnotobiotic mice (Chapter 3, manuscript in preparation).

B3glat5 encodes an essential enzyme for the biosynthesis of type 1 Lewis antigen, including Le^b, Le^a and sLe^a antigen, within cells of the gastrointestinal epithelium and pancreatic tissue.[21] *Fut2* is also involved in the biosynthesis of Le^b antigen.[34] Le^b antigen on gastric epithelial surface has been identified as a primary receptor for the attachment of *Helicobacter pylori* to gastric epithelial cells by binding to the BabA adhesin on the surface of the bacteria.[35] This receptor-ligand interaction is considered to be critical in facilitating the initial colonization and long-term persistence in the stomach. *Ceacam12* encodes a glycoprotein which belongs to the carcinoembryonic antigen (CEA) family, a subfamily of the immunoglobulin (Ig) superfamily.[36] Upregulation of genes within the Ceacam family may provide a mechanism that facilitates bacterial colonization effectively counteracting epithelial exofoliation.[37, 38] Alteration of mucosal epithelial cell glycosylation has been shown to affect bacterial adherence and invasion contributing to the development of IBD.[39-41] Collectively, the upregulation of genes involved in glycoprotein synthesis or surface remodeling of epithelial cells might affect bacterial adherence to the mucosa and, therefore, bacterial-host cross-talk which potentially increases host susceptibility to colitis. Interestingly, in comparison to 30 and 45 DPI, the highest expression levels of genes involved in glycoprotein synthesis at 15 DPI correlate with the greatest gut *H. bilis* numbers at the same time point (Supplemental figure 2A).

Of the downregulated genes identified, *Ces1* and *Ces3* encode carboxylesterases involved in the hydrolysis of ester- and amide-bond-containing drugs such as opiods.[42] *Cyp4b1*, one member of cytochrome P45 gene family, participates in the metabolism of

certain xenobiotic that are protoxic, including valproic acid, 3-methylindole, 4-ipomeanol, 3-methoxy-4-aminoazobenzene, and numerous aromatic amines.[43] *Gstt1*, a subunit of glutathione S-transferases, is involved in mucosal protection against toxins and its polymorphism had been associated with inflammatory bowel disease patients.[44] While the functions of *Ugt8a* is intestine is unknown, UDP-galactosyltransferases have been shown to be involved in detoxification of exogenous compounds such as phenols, flavones.[45] In addition, the detoxification-associated transcription factor *Car(Nr1i3)* was downregulated after *H. bilis* colonization. Agonists of detoxification-associated transcription factors have been shown to ameliorate the DSS-induced colitis,[46] indicating that regulation of mucosal detoxification processes by targeting transcriptional factors may be therapeutically useful for IBD patients.[46] In addition, cytolethal distending toxin (CDT) was considered as an important virulence factor of *H. bilis*.[47] It will be of interest to examine if the defective mucosal detoxification can affect the host ability to effectively detoxify or eliminate this toxin.

In addition to forming a physiological barrier between luminal bacteria and immune cells within the lamina propria, intestinal epithelial cells participate in the process of detoxification and biotransformation of drugs and xenobiotics.[27] Loss or dysregulation of detoxification and biotransformation processes by the intestinal epithelium may contribute to the initiation and progression of IBD.[27-29] Downregulation of detoxification genes has been described for several animal models of IBD including DSS-induced colitis, TCR$\alpha^{-/-}$, and transfer of CD45RB^{hi} T cells and in human patients.[48, 49, 29] It is still unknown whether dysregulated detoxification functions are the causal factor of IBD or arise secondary to inflammation. However, *Mdr1a*$^{-/-}$ mice, which exhibit defective drug transporter gene and detoxification functions, spontaneously develop colitis,[50] supporting the former hypothesis. Furthermore, *H. bilis* colonization has been shown to accelerate the development of colitis in *Mdr1a*$^{-/-}$ mice [6-7] indicating *H. bilis* might exacerbate the defective detoxification functions of intestinal epithelial cells. Likewise, mice were shown to be more susceptible to DSS-induced colitis following suppression of another colonic detoxification enzyme,glutathione S-transferase.[51, 52] Thus, the persistent downregulation of several genes

involved in the different stages of epithelial cell detoxification following colonization with *H. bilis* might predispose the host to the onset of colitis.

Defective or altered mucosal barrier function has been widely considered to be one of the factors contributing to the development of IBD.[1] Additionally, genes associated with mucosal barrier function such as *Mdr1a, Slc22a4, Slc22a5* (solute carrier family 22 member 4 and 5), and *Dlg5* (discs, large homolog 5) have been identified and investigated as potential IBD susceptibility genes.[53, 54] In the present studies, several genes associated with glycoprotein synthesis and detoxification such as *Fut2, B3galt5, Ceacam12, Cyp4b1* and *Ugt8a* were shown to be similarly affected in mice following onset of DSS-induced colitis or *B. hyodysenteriae*-induced colitis, indicating the importance of these genes in maintaining mucosal homeostasis and barrier function independent of the colitis model studied. In contrast, inoculation of the gnotobiotic mice with killed *H. bilis* did not induce changes in mucosal gene expression (data not shown). In conclusion, this study provides insights respective to changes in mucosal gene expression following colonization of the host with *H. bilis.* Furthermore, these results indicate that *H. bilis* colonization perturbed mucosal homeostasis increasing the susceptibility of the host to the development of colitis (Chapter 3, manuscript in preparation). Although *H. bilis* colonization did not result in significant histopathological lesions, the cumulative effects of *H. bilis* colonization on mucosal gene expression (e.g., increased protein glycosylation and dysregulation of detoxification) would likely predispose the host to a more severe inflammatory response to a subsequent colitic insult, which is consistent with a "multiple hit hypothesis" for the development of IBD.[33]

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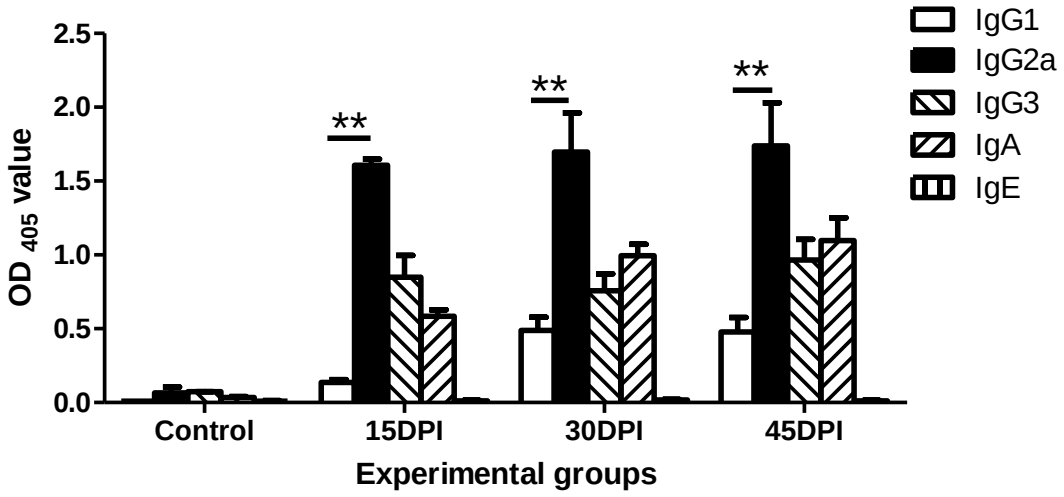
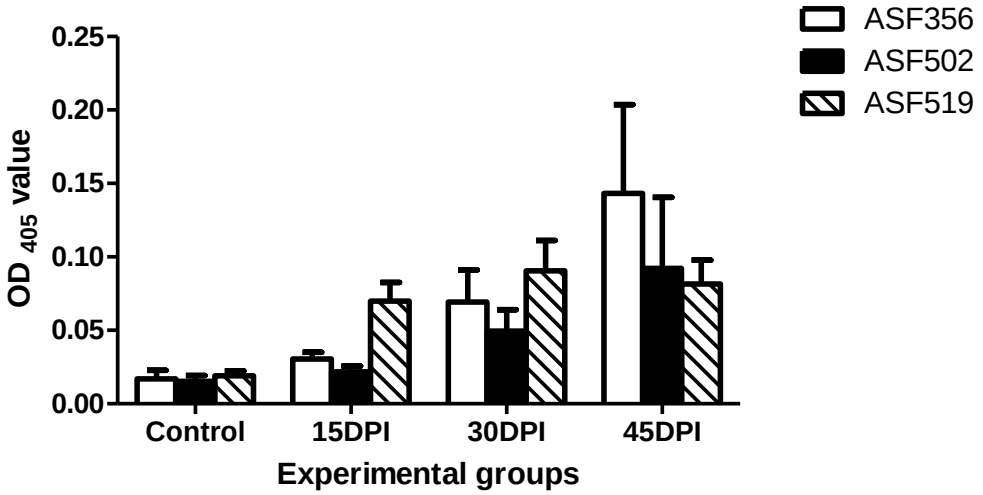
Supplemental Data:

(A)

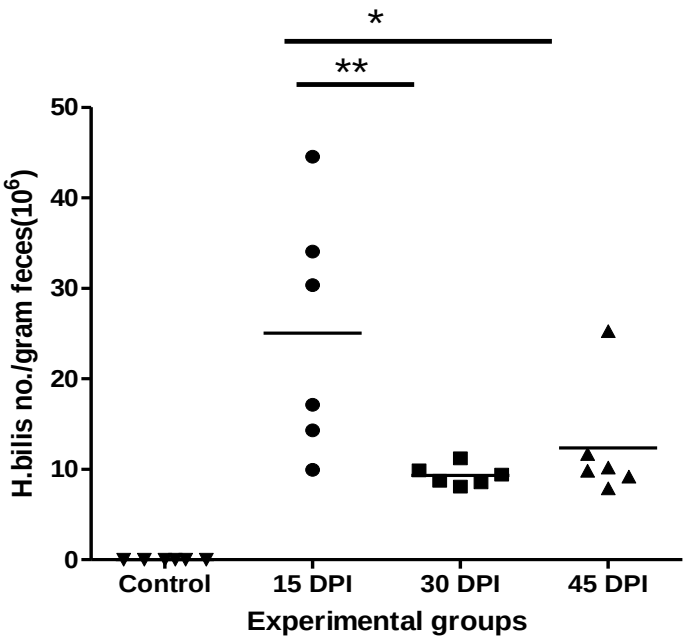


(B)

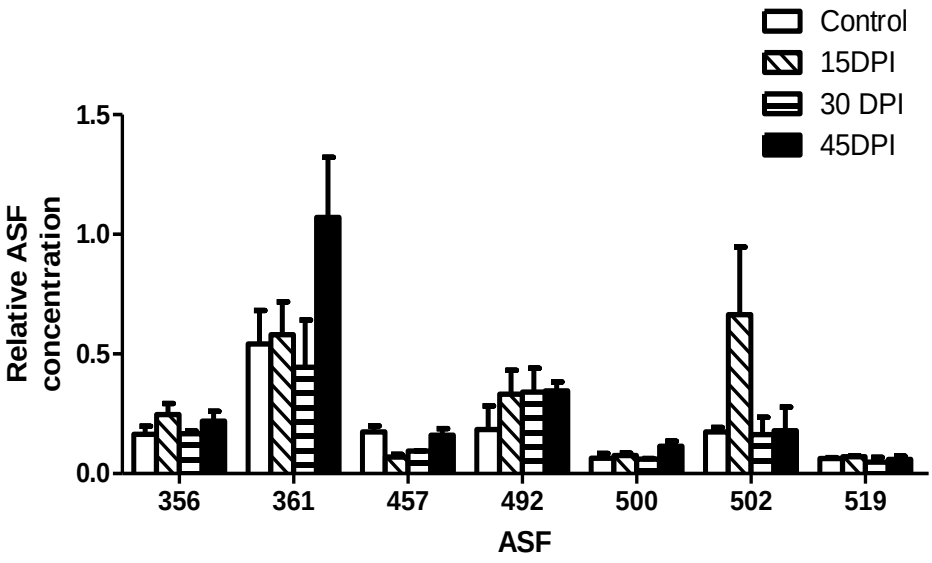


Supplemental Figure 1. Measurement of antibody responses to *Helicobacter bilis* and altered Schadler's flora (ASF) of gnotobiotic mice at 15, 30 and 45 days after the colonization of *Helicobacter bilis*. Sera samples collected at necropsy were diluted and added into the 96-well microtiter plates coated with the whole cell lysates of *H. bilis* or ASF. Isotype specific antibody responses were measured by enzyme-linked immunosorbent assay (ELISA). n = 6 mice. ** $P<0.01$. (A) Antibody responses to *H. bilis.* (B) IgG_{2a} –specific antibody responses selected members of ASF. *H. bilis* colonization induced antibody responses to both *H. bilis* and ASF.

(A)


*
**
50
40
30
20
10
0
H.bilis no./gram feces(10^6)
Control
15 DPI
30 DPI
45 DPI
Experimental groups

(B)


Control
15DPI
30 DPI
45DPI
1.5
1.0
0.5
0.0
Relative ASF concentration
356
361
457
492
500
502
519
ASF

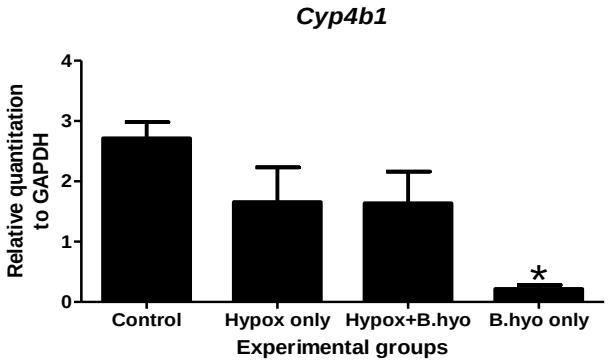
Supplemental Figure 2. Quantitation of cecal *Helicobacter bilis* and altered Schadler's flora (ASF) of gnotobiotic mice at 15, 30 and 45 days after the colonization of *Helicobacter bilis*. DNA was extracted from cecal contents. Quantitative PCR protocols were developed and optimized to detect the 16S rRNA sequence of *H. bilis* and each ASF bacterium (Riley LK, et al, 1996. Sarma-Rupavtarm RB et al, 2004). *Helicobacter bilis* numbers were normalized to per gram cecal contents. Previously published rRNA operon numbers for each ASF strain were used to correlate qPCR results to bacterial cell numbers. n=6 mice. * $P<0.05$, ** $P<0.01$. (A) The number of *H. bilis* (B) The number of ASF. The number of *H. bilis* peaked at 15 DPI and went down / stabilized at 30 and 45 DPI. *H. bilis* colonization did not alter the number of ASF in the gut.

References:

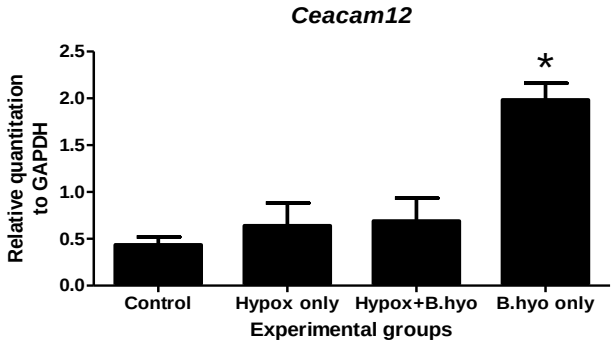
Riley LK, Franklin CL, Hook RR Jr, et al. Identification of murine *helicobacters* by PCR and restriction enzyme analyses. *J Clin Microbiol* 1996; 34:942–946

Sarma-Rupavtarm RB, Ge Z, Schauer DB,et al. Spatial distribution and stability of the eight microbial species of the altered schaedler flora in the mouse gastrointestinal tract. *Appl Environ Microbiol.* 2004; 70(5):2791-800.

(A)



(B)



Supplemental Figure 3. The effects of hypoxoside treatment on the expression levels of genes *Cyp4b1* and *Ceacam12* in ceca of C3H mice infected with *Brachyspira hyodysenteriae*. Mice were orally treated with hypoxoside for seven days prior to *B. hyodysenteriae* infection and the treatments continued daily for seven days postinfection (DPI). Seven days after *B. hyodysenteriae* infection, mice were necropsy and ceca tissues were collected. RNA were extracted from ceca tissues and converted into cDNA. cDNA were amplified via quantitative PCR using the gene specific primers. The quantitation of specific genes was normalized into glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Control: mice didn't receive *B. hyodysenteriae* challenge or hypoxoside treatment; Hypox only: mice received hypoxoside treatment but not *B. hyodysenteriae* challenge; Hypox + B. hyo: mice received both hypoxoside treatment and *B. hyodysenteriae* challenge; B. hyo only: mice received *B. hyodysenteriae* challenge but not hypoxoside treatment. n=6 mice. * P <0.05 vs. all other groups. *Cyp4b1*: Cytochrome P450, family 4, subfamily b, polypeptide 1. *Ceacam12*: CEA-related cell adhesion molecule 12.

The list of all differentially expressed genes in cecal mucosa of gnotobiotic mice following the colonization of *Helicobacter bilis*.

The list of differentially expressed genes induced by *Helicobacter bilis* colonization
(115 genes, $q \leq 0.05$, fold change over control ≥ 2 or ≤ -2)

Upregulated gene list (1)

			Fold change over control				
GeneName	Gene Title	Gene Symbx	15DPI	30DPI	45DPI	Genebank ID	qvals
1423934_at	---	---	2.3	1.0	1.4	BC022950	0.050
1455151_at	A kinase (PRKA) anchor protein (yotiao) 9	Akap9	2.1	0.8	1.1	C79026	0.040
1419136_at	aldo-keto reductase family 1, member C18	Akr1c18	3.5	1.3	1.3	NM_134066	0.048
1420946_at	alpha thalassemia/mental retardation syndrome X-linked homolog (human)	Atrx	2.0	1.4	1.4	BB825830	0.042
1420947_at	alpha thalassemia/mental retardation syndrome X-linked homolog (human)	Atrx	3.9	1.4	1.5	BB825830	0.042
1425382_a_at	aquaporin 4	Aqp4	34.6	1.7	2.3	U48399	0.037
1434449_at	aquaporin 4	Aqp4	26.9	1.4	2.1	BB193413	0.033
1447745_at	aquaporin 4	Aqp4	9.2	1.1	1.3	AW489155	0.037
1444355_at	ATPase, aminophospholipid transporter (APLT), class I, type 8A, member 1	Atp8a1	2.7	1.4	1.5	AW125445	0.044
1448730_at	carboxypeptidase A3, mast cell	Cpa3	8.1	1.6	1.9	NM_007753	0.050
1417268_at	CD14 antigen	Cd14	2.3	1.2	1.1	NM_009841	0.031
1424509_at	CD177 antigen	Cd177	22.4	2.6	2.8	BC027283	0.037
1450136_at	CD38 antigen	Cd38	8.4	2.2	2.7	NM_007646	0.014
1433741_at	CD38 antigen	Cd38	7.2	2.0	2.7	BB256012	0.014
1416777_at	CEA-related cell adhesion molecule 12	Ceacam12	101.9	14.1	17.2	NM_026087	0.044
1436895_at	centaurin, delta 1	Centd1	4.9	1.8	1.9	BB182934	0.050
1455019_x_at	cytoskeleton-associated protein 4	Ckap4	4.3	1.8	1.9	BB818012	0.018
1426754_x_at	cytoskeleton-associated protein 4	Ckap4	3.2	1.4	1.5	BB312117	0.018
1452181_at	cytoskeleton-associated protein 4	Ckap4	3.3	1.4	1.5	BB312117	0.042
1439825_at	deltex 3-like (Drosophila)	Dtx3l	2.5	1.7	1.7	BB705351	0.050
1422137_at	dual oxidase maturation factor 2	Duoxa2	14.2	3.6	3.1	NM_025777	0.014
1425225_at	Fc fragment of IgG, low affinity IIIa, receptor	Fcgr3a	2.2	1.4	1.4	BC027310	0.046
1421775_at	Fc receptor, IgE, high affinity I, alpha polypeptide	Fcer1a	11.3	1.3	1.6	NM_010184	0.044
1434862_at	fucosyltransferase 2	Fut2	19.1	3.2	3.9	AV371838	0.024
1450246_at	fucosyltransferase 2	Fut2	9.3	2.0	2.0	NM_018876	0.014
1425075_at	GATA zinc finger domain containing 2B	Gatad2b	2.5	1.4	1.3	AF411837	0.050
1417898_a_at	granzyme A	Gzma	29.9	1.6	1.5	NM_010370	0.014
1419060_at	granzyme B	Gzmb	34.3	1.8	2.2	NM_013542	0.026
1420499_at	GTP cyclohydrolase 1	Gch1	4.4	1.7	1.5	NM_008102	0.046
1420549_at	guanylate nucleotide binding protein 1	Gbp1	4.4	1.5	1.7	NM_010259	0.014
1435906_x_at	guanylate nucleotide binding protein 2	Gbp2	3.7	1.5	1.7	BE197524	0.037

(Continued)

Upregulated gene list (2)			Fold change over control				
GeneName	Gene Title	Gene Symbo	15DPI	30DPI	45DPI	Genebank ID	qvals
1422612_at	hexokinase 2	Hk2	3.1	1.6	1.8	NM_013820	0.042
1445597_s_at	HRAS like suppressor 3	Hrasls3	2.1	1.1	1.2	BB404920	0.047
1427851_x_at	immunoglobulin heavy chain (J558 family)	Igh-VJ558	8.8	5.8	4.4	AF065324	0.014
1451951_at	Immunoglobulin kappa chain variable 14-111	Igkv14-111	15.4	5.4	6.1	U62386	0.048
1426263_at	immunoglobulin superfamily, member 4C	Igsf4c	3.7	1.1	1.2	AY059394	0.014
1417292_at	interferon gamma inducible protein 47	Ifi47	4.7	2.2	2.9	NM_008330	0.042
1418230_a_at	LIM and senescent cell antigen-like domains 1	Lims1	2.7	1.5	1.3	BC005621	0.023
1417185_at	lymphocyte antigen 6 complex, locus A	Ly6a	13.6	1.4	2.3	BC002070	0.037
1424349_a_at	lysophosphatidylglycerol acyltransferase 1	Lpgat1	2.2	1.3	1.2	BE987427	0.050
1452037_at	mannoside acetylglucosaminyltransferase 2	Mgat2	2.6	1.4	1.5	AI481328	0.037
1422352_at	mast cell protease 1	Mcpt1	54.8	2.9	3.3	NM_008570	0.038
1449989_at	mast cell protease 2	Mcpt2	35.6	2.6	2.8	NM_008571	0.037
1450538_s_at	mast cell protease 9 /// chymase 2, mast cell	Mcpt9 /// Cm	12.4	1.5	1.3	AY007568	0.009
1425783_at	membrane targeting (tandem) C2 domain containing 1	Mtac2d1	2.4	1.2	1.0	AB062282	0.037
1443264_at	membrane-spanning 4-domains, subfamily A, member 2	Ms4a2	3.2	1.1	1.1	AV241486	0.037
1419599_s_at	membrane-spanning 4-domains, subfamily A, member 6D	Ms4a6d	4.7	2.4	2.6	NM_026835	0.048
1450537_at	midline 2	Mid2	3.2	1.3	1.2	AF196480	0.044
1427398_at	mucin 4	Muc4	2.9	0.9	1.2	AF218265	0.044
1416069_at	phosphofructokinase, platelet	Pfkp	2.0	1.2	1.2	NM_019703	0.046
1417814_at	phospholipase A2, group V	Pla2g5	16.1	1.8	2.3	AF162713	0.046
1451564_at	poly (ADP-ribose) polymerase family, member 14	Parp14	2.2	1.4	1.6	BC021340	0.044
1450035_a_at	PRP40 pre-mRNA processing factor 40 homolog A (yeast)	Prpf40a	3.0	1.6	1.6	BG064340	0.050
1450271_at	PTK6 protein tyrosine kinase 6	Ptk6	4.0	1.3	1.1	NM_009184	0.046
1448449_at	receptor-interacting serine-threonine kinase 3	Ripk3	3.3	1.5	1.4	NM_019955	0.040
1454254_s_at	RIKEN cDNA 1600029D21 gene	1600029D21	558.1	11.1	15.7	AK002767	0.044
1423933_a_at	RIKEN cDNA 1600029D21 gene	1600029D21	348.3	9.9	16.2	BC022950	0.037
1438555_x_at	RIKEN cDNA 4933405I11 gene /// mucin 4	4933405I11I	7.8	1.1	1.6	BB532597	0.047
1450891_at	signal recognition particle 19	Srp19	2.2	1.3	1.3	W08076	0.037
1435529_at	similar to interferon-induced protein with tetratricopeptide repeats 1 /// similar to ir	LOC667373	16.6	5.1	7.3	BM245961	0.018
1448025_at	similar to SIRP beta 1 isoform 2	LOC668101	4.0	1.6	2.2	AI662854	0.047
1418857_at	solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 2	Slc13a2	2.4	1.4	1.4	BC013493	0.041
1419570_at	solute carrier family 28 (sodium-coupled nucleoside transporter), member 3	Slc28a3	2.4	1.3	1.2	NM_022317	0.040
1417300_at	sphingomyelin phosphodiesterase, acid-like 3B	Smpdl3b	4.9	1.5	2.2	NM_133888	0.040
1452885_at	splicing factor, arginine/serine-rich 2, interacting protein	Sfrs2ip	4.5	1.9	1.8	AK012092	0.046

(Continued)

Upregulated gene list (3)			Fold change over control				
GeneName	Gene Title	Gene Symb	15DPI	30DPI	45DPI	Genebank ID	qvals
1451342_at	spondin 1, (f-spondin) extracellular matrix protein	Spon1	4.2	1.6	2.0	BC020531	0.044
1438910_a_at	stomatin	Stom	3.8	1.1	1.3	BB782444	0.046
1419097_a_at	stomatin	Stom	3.8	1.0	1.1	AF093620	0.050
1422188_s_at	T-cell receptor gamma chain /// T cell receptor gamma chain /// region containing	Tcrg /// LOC	4.6	1.3	1.1	NM_011558	0.044
1417500_a_at	transglutaminase 2, C polypeptide	Tgm2	2.4	1.1	1.2	BC016492	0.050
1426004_a_at	transglutaminase 2, C polypeptide	Tgm2	2.3	1.0	1.2	AF114266	0.046
1456112_at	translocated promoter region	Tpr	3.3	1.5	1.7	AW554765	0.050
1420412_at	tumor necrosis factor (ligand) superfamily, member 10	Tnfsf10	2.5	1.3	1.2	NM_009425	0.050
1428397_at	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 5	B3galt5	102.1	7.3	7.2	BB430637	0.044
1428398_at	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 5	B3galt5	92.2	6.1	6.0	BB430637	0.014
1450528_at	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 5	B3galt5	33.1	2.2	1.9	NM_033149	0.021
1459801_at	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 5	B3galt5	3.3	1.3	1.2	BB189298	0.014
1436321_at	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 7	B3gnt7	41.6	3.1	4.3	BF578266	0.014
1429947_a_at	Z-DNA binding protein 1	Zbp1	10.6	2.5	3.9	AK008179	0.046
1443993_at	zinc finger CCCH type containing 12A	Zc3h12a	3.3	1.3	1.3	BE852171	0.023

Downregulated gene list

GeneName	Gene Title	Gene Symbol	15DPI	30DPI	45DPI	Genebank ID	q values
1450643_s_at	acyl-CoA synthetase long-chain family member 1	Acsl1	-3.0	-1.3	-1.6	BI413218	0.018
1423883_at	acyl-CoA synthetase long-chain family member 1	Acsl1	-2.9	-1.4	-1.5	BC006692	0.046
1449457_at	acyl-CoA thioesterase 12	Acot12	-4.1	-1.4	-1.9	AB078618	0.050
1448813_at	arylacetamide deacetylase (esterase)	Aadac	-3.6	-1.7	-1.5	NM_023383	0.037
1449486_at	carboxylesterase 1	Ces1	-3.0	-1.6	-1.5	NM_021456	0.040
1435371_x_at	carboxylesterase 3	Ces3	-3.2	-1.5	-1.5	AI315015	0.046
1434532_at	cDNA sequence BC035295	BC035295	-2.1	-1.4	-1.5	BB796273	0.044
1416194_at	cytochrome P450, family 4, subfamily b, polypeptide 1	Cyp4b1	-65.6	-2.1	-4.2	NM_007823	0.043
1418767_at	cytochrome P450, family 4, subfamily f, polypeptide 13	Cyp4f13	-2.0	-1.3	-1.5	NM_130882	0.046
1448499_a_at	epoxide hydrolase 2, cytoplasmic	Ephx2	-4.1	-1.9	-1.9	NM_007940	0.050
1455007_s_at	glutamic pyruvate transaminase 2	Gpt2	-3.9	-1.4	-1.7	BI648645	0.050
1418186_at	glutathione S-transferase, theta 1	Gstt1	-2.5	-1.8	-1.6	BC012254	0.038
1419499_at	glycerol-3-phosphate acyltransferase, mitochondrial	Gpam	-3.8	-1.8	-1.7	NM_008149	0.032
1451370_at	hypothetical protein LOC622403	LOC622404	-2.2	-1.5	-1.5	BC027414	0.050
1451547_at	iodotyrosine deiodinase	Iyd	-5.6	-2.3	-1.8	BC023358	0.037
1455883_a_at	leucine rich repeat transmembrane neuronal 1	Lrrtm1	-10.4	-3.6	-2.8	BB269910	0.040
1452624_at	leucine rich repeat transmembrane neuronal 1	Lrrtm1	-2.5	-1.7	-1.8	BM900077	0.024
1420981_a_at	LIM domain only 4	Lmo4	-2.7	-1.3	-1.5	NM_010723	0.046
1449789_x_at	lymphocyte antigen 6 complex, locus G6C	Ly6g6c	-6.1	-1.8	-1.6	AV088850	0.050
1454858_x_at	methyltransferase like 7A	Mettl7a	-2.4	-1.3	-1.3	AV171622	0.023
1434151_at	methyltransferase like 7A	Mettl7a	-2.6	-1.3	-1.2	AV171622	0.044
1437492_at	mohawk	Mkx	-3.1	-1.8	-1.7	BB021019	0.046
1417928_at	PDZ and LIM domain 4	Pdlim4	-2.5	-1.6	-1.6	NM_019417	0.021
1423725_at	plastin 3 (T-isoform)	Pls3	-2.0	-1.3	-1.3	BC005459	0.044
1431126_a_at	RIKEN cDNA 0610011F06 gene	0610011F06Rik	-2.2	-1.5	-1.4	AK002535	0.046
1428915_at	sirtuin 5	Sirt5	-2.1	-1.5	-1.6	AK002609	0.046
1428805_at	solute carrier family 35, member E3	Slc35e3	-4.2	-1.6	-1.4	AI854658	0.048
1451228_a_at	syncollin	Sycn	-3.7	-1.4	-1.1	BC019567	0.018
1434803_a_at	syncollin	Sycn	-3.6	-1.4	-1.1	AV050299	0.021
1455913_x_at	transthyretin	Ttr	-5.0	-1.5	-1.5	AV152953	0.049
1419063_at	UDP galactosyltransferase 8A	Ugt8a	-79.5	-2.3	-2.3	NM_011674	0.037
1417122_at	vav 3 oncogene	Vav3	-2.7	-1.8	-1.8	BC027242	0.048
1448600_s_at	vav 3 oncogene	Vav3	-2.5	-1.6	-1.6	BC027242	0.046
1417900_a_at	very low density lipoprotein receptor	Vldlr	-4.2	-1.7	-2.2	NM_013703	0.044
1422634_a_at	V-set and immunoglobulin domain containing 2	Vsig2	-2.2	-1.3	-1.4	NM_020518	0.040

CHAPTER 3. EFFECTS OF *Helicobacter bilis* COLONIZATION ON MURINE COLITIS INDUCED BY DEXTRAN SODIUM SULFATE

A paper to be submitted to the journal *Clinical & Experimental Immunology*

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ABSTRACT

Colonization with *Helicobacter bilis* has been shown to induce or accelerate the development of colitis in immune-deficient mice and to trigger host immune responses to commensal bacteria in immunocompetent mice. These results indicate that *H. bilis* colonization affects mucosal homeostasis and likely increases host susceptibility to IBD. However, the molecular and cellular mechanisms governing the effects of *H. bilis* colonization on the development of colitis remain unknown. In this study, mice were colonized with *H. bilis* for 6 weeks and then treated with a low dose of dextran sodium sulfate (DSS, 1.5 %) for five days followed by a four day restitution period. Severity of DSS-induced lesions was assessed. Gene expression profiles and mucosal cellular infiltration were characterized. *Helicobacter bilis* colonization increased severity of intestinal inflammatory lesions induced by DSS. Particularly, *H. bilis* colonization activated genes associated with immune cell activation (e.g., *Cd28* and *Tnfsf13b*) and chemotaxis (e.g., *Itgb2*, *Ccl8*, and *Ccr5*). Consistent with these observations, increased infiltration/proliferation of mucosal and/or MLN T cells, B cells, macrophages, but not neutrophils, were observed in colonic tissue from mice colonized with *H. bilis* and treated with DSS, in comparison to mice treated with DSS alone. In addition, *H. bilis* colonization downregulated the expression levels of the detoxification-associated genes, *Cyp4b1* and *Ugt8a*. Activation/recruitment of

immune cells and downregulation of detoxification-associated genes induced by *Helicobacter bilis* colonization might be associated with the increased host susceptibility to DSS colitis.

Keywords: *Helicobacter bilis*, colitis, dextran sodium sulfate, cell infiltration, detoxification

INTRODUCTION

Accumulating evidence from animal models and patients with inflammatory bowel disease (IBD) (i.e., Crohn's disease and ulcerative colitis) demonstrate a pivotal role for bacteria in the induction or exacerbation of enteric inflammation [1]. For example, colitis is often more severe in regions heavily colonized with bacteria and antibiotic therapy has been used to treat Crohn's disease [2, 3]. More convincingly, IL-$2^{-/-}$, IL-$10^{-/-}$, TCR$\alpha^{-/-}$ mice, and HLA-B27 transgenic rats only develop colitis when colonized with an intestinal microflora [4, 5]. The current etiological hypothesis supports the contention that aberrant immune responses to the microflora, along with genetic susceptibility of the host, are major contributors to the development of IBD [6].

However, not all bacteria have the same effect on or potential to induce the development of IBD. For instance, *Bacteriodes vulgatus* is preferentially able to induce colitis in HLA-B27 transgenic rats, whereas other bacteria such as *Escherichia coli* stimulate no inflammatory response in this model of colitis [7]. Enterohepatic *Helicobacter* spp (EHS), a bacterial genus naturally infecting many laboratory rodents, has been linked to the induction of colitis and has significantly contributed to the morbidity of immunodeficient mice [8]. In addition, EHS have been isolated from a subset of IBD patients and has also been shown to cause bacterial-induced colitis [9-11]. More importantly, emerging evidence suggests that inflammatory responses to EHS infections alter host responses to other experimental stimuli, although *Helicobacter*-associated disease is not clinically observable in most immunocompetent rodents [12].

Helicobacter bilis, one of the most prevalent EHS in laboratory animals, has been shown to induce or accelerate colitis in immunodeficient rodents including *SCID*, multiple drug resistance-deficient (Mdr1a$^{-/-}$), IL-$10^{-/-}$, or TCR$\alpha^{-/-}$ mice and athymic nude rats [13-17]. Furthermore, *H. bilis* colonization has been shown to induce differential host immune

responses including antibody and cytokine responses against antigens derived from the resident flora in immunocompetent mice [18-20]. These results suggest that introduction of *H. bilis* might disrupt mucosal homeostasis, thus, increasing host susceptibility to IBD. However, the molecular and cellular mechanisms mediating the increased sensitivity to colitis following *H. bilis* colonization remain unknown.

Common features of intestinal inflammation, such as IBD, include the massive infiltration of leukocytes (e.g., granulocytes, macrophage, and lymphocytes) into the intestinal mucosa and the production of cytokines (e.g., IL-1β, TNFα and IFNγ) and chemokines (e.g., CXCL8, CCL5, CCR5) [21]. However, there are important differences in immunopathological features between different forms of intestinal inflammation. For example, histological features of Crohn's disease include the development of mucosal granulomas that are characterized by the presence of compact macrophages, giant cells, and epithelioid cells surrounded by a marked infiltration of lymphoid cells, plasma cells, and other inflammatory cells. In contrast, ulcerative colitis is characterized by crypt abscesses that are composed of infiltrating neutrophils and sloughing epithelium [22]. In addition, Crohn's disease is often associated with a TH1-type cytokine profile; on the other hand, ulcerative colitis appears to present with a TH2-type profile [22].

In the present study, we demonstrated that *H. bilis* colonization increased the severity of colitis induced by dextran sodium sulfate (DSS) and further characterized the changes in the mucosal gene expression profile and inflammatory cell infiltration following *H. bilis* colonization that might increase the host's susceptibility/sensitivity to colitis. Our results suggest that *Helicobacter bilis* colonization evoked significant increases in genes associated with T cell and B cell activation (e.g., *Cd28* and *Tnfsf13b* expression), macrophage infiltration, and negatively affected epithelial detoxification activities (e.g., *Cyp4b1* and *Ugt8a*). Thus, *H. bilis* colonization affected mucosal homeostasis by aggravating mechanisms that enhanced the sensitivity or susceptibility to enteric inflammation. The identification of host factors that predispose to the onset of colitis following colonization with bacterial provocateurs such as *H. bilis* will advance the understanding of mechanisms of colitis and lead to new intervention strategies for the prevention and control of colitis.

MATERIAL AND METHODS

Mice and bacteria. Eight to nine week old, gnotobiotic C3H/HeN:Tac mice colonized with the altered Schaedler's flora (ASF) were obtained from Taconic Farms (Germantown, NY) and gnotobiotically maintained at Iowa State University. Members of the ASF include ASF356, *Clostridium cluster XIV;* ASF360, *Lactobacillus acidophilus;* ASF361, *Lactobacillus murinus;* ASF457, *Mucispirillum schaedleri;* ASF492, *Eubacterium plexicaudatum;* ASF500, *low-G+C content-positive bacteria;* ASF502, *Clostridium cluster XIV*; and ASF519, *Bacteroides distasonis*. All animal-related procedures were approved by the Animal Care and Use Committee at Iowa State University.

Helicobacter bilis was cultivated and harvested as previously described [18]. Mice were orally colonized once daily over three consecutive days with approximately 10^8 CFU of *H. bilis* per day.

Experimental design. At six weeks post-infection with *H. bilis,* mice were exposed to drinking water containing 1.5% DSS (M.W. 36, 000 - 50, 000, MP Biomedicals, Solon, OH) for five days, followed by four days with regular drinking water. Mice (n = 10 to 14 per group) were divided into two groups: DSS group in which mice were treated with DSS alone and *H. bilis* + DSS group in which mice were colonized *H. bilis* and treated with DSS. At the termination of the experiments, mice were euthanized and the ceca/colon, MLN, and sera were collected for further analysis. Control normal mice and mice that colonized with *H. bilis* alone were also included in this study.

Gross inflammatory lesion score and histology. Inflammatory lesion scores were evaluated as previously described [18]. Briefly, gross inflammatory lesions were scored 0 to 3 as follows: cecal atrophy and excess intraluminal mucus = 3; atrophy and excess intraluminal mucus localized to the cecal apex = 2; increased cecal mucus with no evidence of atrophy = 1; and no gross lesions = 0. For microscopic lesions, cecal and colonic tissues were formalin-fixed, embedded in paraffin, routinely processed, sectioned at 5 μm, and stained with hematoxylin and eosin. Stained tissue sections were scored by a pathologist (Dr. J. M. Hostetter, Department of Veterinary Pathology, Iowa State University) that was blinded to the treatment groups. Histological scores were based on the severity of mucosal epithelial damage, degree of lamina proprial cellular infiltrate, and architectural distortion [18].

Affymetrix oligonucleotide array analysis. RNA samples extracted from colonic tissues were submitted to the Iowa State University Genechip Facility (Ames, IA). Labeling of samples, hybridization, staining, and scanning were performed according to protocols outlined in the Affymetrix Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). Affymetrix Mouse genome 430 2.0 arrays were used for these experiments.

Fluorescence intensity values (.CEL files) generated from hybridized and labeled genechips were analyzed using R software (http://www.r-project.org) and a Bioconductor analysis package (http://www.bioconductor.org). Data were normalized using Robust Multi-array Average (RMA) [23]. False discovery rate (*q* value) of normalized data was computed using Storey and Tibshirani's default method [24]. Differentially expressed genes were statistically filtered using a *q* value ≤ 0.05 and a estimated fold change ≥ 2 or ≤ -2. Assignments of filtered genes to functional categories were performed by using Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics resource 2007 (National Institutes of Health, Bethesda) [25]. Fisher exact tests were performed to calculate *P* values and determine which particular annotation categories were over-represented.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR). Total RNA was isolated from colonic tissues using the RNeasy Mini Kit (Qiagen, Valencia, CA) and then reverse transcribed into cDNA by SuperScript™ III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). The cDNA was amplified using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen). The PCR conditions used for these studies were as follows: 95 ℃ for 10 minutes, followed by 40 cycles of amplification (95 ℃ for 15 seconds, 60 ℃ for 60 seconds). The relative mRNA quantities were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific mRNA. The quantitative PCR was performed using a Rotor-Gene 3000 (Corbett Research, Mortlake**,** Australia). Standard curves for the selected genes and GAPDH were made using serial dilutions of cDNA. The correct sizes of the amplified PCR products were confirmed by agarose gel electrophoresis. The primer sequences are available upon request.

Myeloperoxidase (MPO) Assay. The assay measuring MPO was modified from a previously published method [26]. Briefly, colonic tissue samples were collected, homogenized, and sonicated in phosphate buffered saline (PBS) containing 0.1 mM

phenylmethanesulphonylfluoride (PMSF). The resulting lysate was centrifuged and the supernatant was aliquoted into a microtiter plate. MPO activity was determined colorimetrically using 3,3', 5,5'-tetramethylbenzidine(TMB) as a substrate at an optical density of 405 nm. Serial dilutions of freshly prepared neutrophils recovered from murine peripheral blood were used to generate standard curves. The MPO levels were normalized based on the total protein of each sample.

F4/80$^+$ macrophage immunohistochemistry. Macrophages were identified using the monoclonal antibody F4/80, a pan-surface marker on murine macrophages. Paraffin-embedded colon tissue sections were deparaffinized, rehydrated, and digested with a 2% protease (Sigma, St Louis, MO) in 0.05 M Tris buffer(PH=7.6) for 20 minutes. After blocking with normal rat sera for 20 minutes, the sections were incubated with 0.5 μg/ml affinity purified anti-mouse F4/80 monoclonal antibody (clone BM8, eBioscience, San Diego, CA) overnight at 4 ℃. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 20 minutes. Subsequently, the sections were incubated with Biotin-SP-AffiniPure F(ab')2 Fragment Goat Anti-Rat IgG (H+L) (Jackson Immunoresearch Laboratory, West Grove, PA) for 30 minutes and HRP-Streptavidin for 15 minutes. The sections were then incubated with a diaminobenzidine (DAB) substrate to reveal brown/black staining at the site of HRP localization. After staining, the sections were washed in distilled water, counterstained with hematoxylin, and mounted. Stained sections were photographed under a microscope.

Semiquantitative approach as previously described [27] was used to score the frequency of positive cells. The immunoreactivity of the stains was scored by the following scale: 0=no immunoreactive cells, 1=rare immunoreactive cells, 2=scattered immunoreactive cells, 3= clusters of immunoreactive cells, 4=nearly diffuse staining.

Lamina propria lymphocytes (LPL) Isolation. The technique used for LPL isolation was modified from a previously described method [28]. Cleansed whole colons were cut into 1 cm pieces and incubated with 20 ml Hanks' Balanced Salt Solution (HBSS) containing 1.25 μM ethylenediaminetetraacetic acids (EDTA) in a shaking water bath at 37 ℃ for 18 minutes to release intraepithelial lymphocytes and epithelial cells. After washing, colonic tissues were incubated with cell culture medium containing 0.5 mg/ml DNAse I collagenase for 20 min with agitation. At the end of the agitation, the tissues were subjected to further mechanical

disruption by aspirating up and down using a 1 ml syringe. The resulting cell suspensions were filtered through sterile gauze to remove large debris. After washing, cells were layered onto a 30:70 % Percoll gradient. Cells were centrifuged at 2,200 $\times g$ at room temperature for 20 min. The LPLs collected from the 30:70 interface were washed and maintained on ice until used.

Flow cytometry. Lamina propria lymphocytes and MLN cell suspensions were incubated with mAbs 17A2 (anti-CD3 mAb; PharMingen Franklin Lakes, NJ), 6D5 (anti-CD19 mAb, Southern Biotechnology, Birmingham, AL) and IgG isotype controls on ice for 20 minutes and then washed. The final concentration of all antibodies used in these studies was 1 μg/ml. Two color flow cytometric analyses were performed using a BD FACScan flow cytometer and CellQuest analysis software. Total CD3 and CD19 positive cells were calculated using the following formula: Total specific cells= (total MLN or LPL cells per mouse $\times$ percentage of specific cells determined by flow cytometry).

Cell proliferation. Antigen presenting cells (APC) were prepared as follows: single cell suspensions of spleen cells (2×10^7/ml) from healthy C3H mice were incubated with whole cell sonicates of pooled ASF antigens (200 μg/ml), *Helicobacter bilis* (200 μg/ml), or cell medium alone overnight at 37 ℃. The cell suspension was then treated with mitomycin C (50 μg/ml) for 30 minutes and then washed five times with medium.

Single cell suspensions (2×10^6 /ml) of MLN cells were co-cultured with antigen-pulsed APC preparation (2×10^6 /ml) at 37 ℃ for five days. Cellular proliferation was assessed by the addition of ^{3}H-thymidine during the last 8 hours of the culture period and proliferation (i.e., ^{3}H-thymidine incorporation) was measured using a liquid scintillation counter.

Detection of antigen-specific serum antibodies. Antigen-specific serum antibodies were measured by ELISA as previously described [19]. Briefly, wells of 96-well microtiter plates (Immulon II) were coated with 10 μg/ml sonicated whole cell lysates of *H. bilis* and each of the individual ASF. After blocking the wells with 1 % gelatin, diluted serum (1:250) was added into each well and incubated at 4 ^{o}C overnight. Then, alkaline phosphatase conjugated goat anti-mouse IgG_1, IgG_{2a}, IgA, or IgE (for ASF analysis, only anti-IgG_1 and anti-IgG_{2a} were used) (Southern Biotech, Birmingham, AL) were added to one set of triplicate samples and incubated for 4 hours at room temperature. Colorimetric reactions

were developed using p-nitrophenyl phosphate at room temperature. Optical densities were measured at 405 nm using a microtiter plate reader.

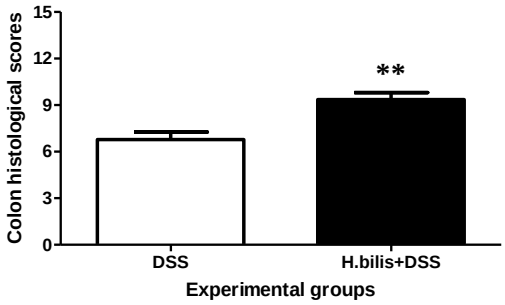
Statistical analysis. The results are expressed as mean ± standard error (SE). The gross and histological lesions were analyzed using the wilcoxon rank-sum test. Other data are analyzed by student t-test or analysis of variance (ANOVA).

RESULTS

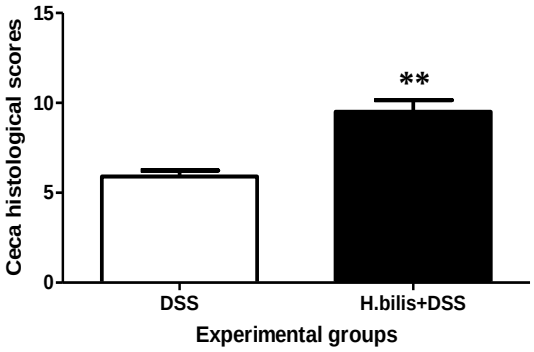
***Helicobacter bilis* colonization increased severity of intestinal inflammatory lesion induced by dextran sodium sulfate.** *Helicobacter bilis* has been shown to induce/accelerate the development of colitis in immunodeficient mice [13-17] and triggered persistent immune responses to commensal bacteria by six weeks post-infection in immunocompetent mice [18, 19], suggesting that colonization affected mucosal homeostasis and increased host susceptibility to colitis. To directly test this hypothesis, *H. bilis* colonized mice were treated with low dose DSS (1.5 %) and the subsequent histological changes were evaluated.

Macroscopically, exposure of the gnotobiotic mice to low dose DSS did not induce observable gross lesions. However, co-treatment of gnotobiotic mice with *H. bilis* and DSS induced significantly higher gross lesion scores than DSS alone ($P < 0.01$). Macroscopic lesions in the co-treatment group were characterized by the presence of increased cecal atrophy and intraluminal mucus (Fig. 1A).

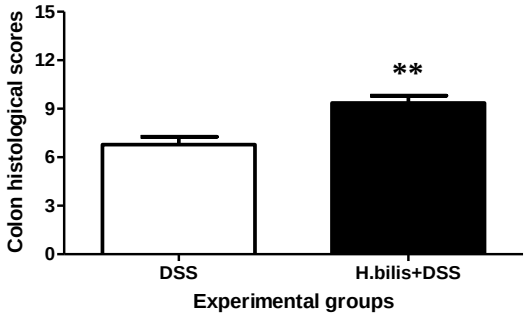
Individually, *H. bilis* colonization and DSS treatment induced subtle increases in the histological score, while the co-treatment of *H. bilis* and DSS induced significantly ($P<0.01$) more severe histological lesions that were characterized by increased mucosal height, inflammatory cell infiltration, mucosal edema, and epithelial erosion (data not shown, Fig. 1). In addition, the severity of histological lesions for each treatment was similarly represented in both ceca and colon (Fig. 1B, C). Notably, more epithelial ulceration was observed in mice colonized with *H. bilis* and treated with DSS, in comparison to mice treated with DSS alone (Fig. 1D). Collectively, *H. bilis* colonization significantly increased the sensitivity of the host to DSS-induced inflammation.



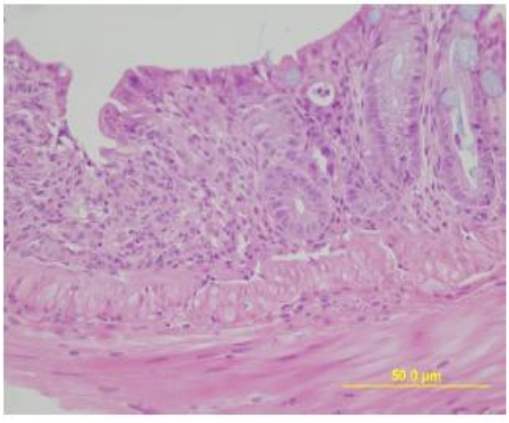
(A) Cecal gross lesions

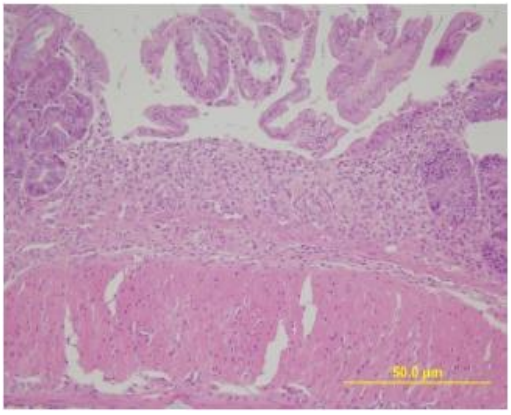


(B) Cecal histological lesions



(C) Colon histological lesions





DSS *H.bilis* + DSS

(D) Representative photomicrographs

Figure 1. Effect of *Helicobacter bilis* colonization on the severity of macroscopic and microscopic lesions following exposure to dextran sodium sulfate (DSS). Six weeks after *H. bilis* colonization, mice were treated with 1.5% DSS in their drinking water for five days followed by a four day restitution period. Upon termination of the experiment, lesion scores were determined as described in Materials and Methods. (A) Macroscopic cecal lesion scores: the gross lesion scores for control and *H. bilis* colonized mice were 0 ± 0 and 0.92 ± 0.15, respectively ; (B) Cecal histological scores: the cecal lesion scores for control and *H. bilis* colonized mice were 4.33 ± 0.25 and 0.92 ± 0.15, respectively ; (C) Colonic histological scores: the colonic lesion scores for control and *H. bilis* colonized mice were 4.69 ± 0.28 and 6.42 ± 0.44, respectively ;; (D) Representative diagram of histological lesions induced by DSS (top) or *H. bilis* + DSS (bottom). Open bars represent DSS group, filled bars represent *H. bilis* + DSS group. n = 10 to 14 mice per group. ** $P < 0.01$ vs. DSS group.

***H. bilis* colonization upregulated the expression levels of genes associated with immune cell activation and chemotaxis.** To directly assess the molecular effects of *H. bilis* on the DSS-induced colitis, mucosal gene expression profile between DSS group and *H. bilis* + DSS group were compared via Affymetrix genechip. Based upon accepted statistical criteria (q value ≤ 0.05 and a estimated fold change ≥ 2 or ≤ -2), 106 probe sets were defined as upregualted in the *H. bilis* plus DSS treated group in comparison to the group treated with DSS alone. However, no genes were identified to be downregulated using these statistical criteria.

The biological themes of 106 probe sets were analyzed using "biological process" of Gene Ontology (GO) terms in the DAVID bioinformatics resource 2007. The results showed that those genes were strongly associated with biological processes such as "defense response" and "immune response". The immune response-associated genes include genes in the "immune activation", "humoral immune responses" and "chemotaxis" subgroups (Table 1).

Some of the upregulated genes were involved in TCR signaling suggesting increased T cell activation (Table 2). For example, *Cd28* which encodes a co-stimulatory molecule involved in T cell activation/proliferation [29] was found to be upregulated. *Fyb,* which

encodes an adaptor protein that interacts with the Src family tyrosine kinase Fyn [30], and *Lcp2* (referred to as Lcp2 or SLP-76), another essential adaptor protein in the TCR signaling pathway [31], were also upregulated. In addition, integrin beta 2 chain (encoded by *Itgb2/Cd18*) which forms a heterodimer along with integrin alpha L chain (encoded by *ItgaL/Cd11a*) form the co-stimulatory integrin lymphocyte function-associated antigen-1 (LFA-1) [32], was also upregulated. The upregulation of these genes indicates the *H. bilis* colonization may result in the activation of mucosal T cells.

Activation of B cells induced by *H. bilis* colonization was indicated by the upregulation of *Tnf13b* (also named B-cell activating factor, BAFF), which is central to the survival and activation of peripheral B cells [33]. A series of genes associated with immunoglobulin synthesis (e.g., *Igm, Igh-vj558* and *Igj*) were also upregulated in *H. bilis* + DSS groups, in comparison to DSS group (see Supplemental data). In addition, the upregulated genes also include interferon-regulatory factor 4 (*Irf4*) which has been shown to be essential for the development of Th-17 T cells [34].

Helicobacter bilis colonization also induced the upregulation of a series of genes associated with chemotaxis (Table 2). The chemokine, *Ccl8*, plays a role in the recruitment of monocytes, lymphocytes, basophils, and eosinophils [35]. *Ccr5* and *Ccr10* can be expressed on the surface of T cells and/or macrophages and have been shown to be involved with the infiltration of regulatory T cells into sites of mucosal inflammation [36, 37]. Integrin beta 2 chain (encoded by *Itgb2/Cd18*) combines with the alpha M chain (encoded by *Itgam/Cd11b*) to form the integrin macrophage receptor 1(Mac-1), which may be involved in the recruitment of monocytes and neutrophils [38]. Upregulation of these genes suggests *H. bilis* colonization might induce the recruitment of various inflammatory cells or regulatory T cells into intestine mucosa. The gene expression changes of *Cd28, Itgb2,* and *Ccr5* were confirmed by qRT-PCR (Fig. 2).

Take together, the microarray results (*H. bilis* + DSS group vs. DSS group) showed that consistent with the increased severity of intestinal lesions, *H. bilis* colonization upregulated genes involved in activation/recruitment of various immune cells. These results indicate the mucosa of the *H. bilis* colonized mice might be primed and more readily recruit immune cells

into the mucosa. However, it is still unknown what types of immune cells may play critical roles on how *H. bilis* colonization effects the development of colitis.

Table 1. The annotation analysis of mucosal differentially expressed genes derived from a comparison between DSS group and *H. bilis* + DSS group.

Annotation[a, b]	**Gene no.**	**Percentage**	***P* Value**[b]
Defense response	31	33.3%	1.40E-21
Response to biotic stimulus	31	33.3%	3.19E-21
Immune response	27	29.0%	3.13E-19
Immune cell activation	12	12.9%	8.22E-12
Cell activation	12	12.9%	8.77E-12
Response to pest, pathogen or parasite	17	18.3%	1.63E-10
Response to other organism	17	18.3%	2.68E-10
Humoral immune response	10	10.8%	5.66E-09
Organismal physiological process	29	31.2%	8.07E-09
Response to stimulus	31	33.3%	1.21E-08
Response to external stimulus	14	15.1%	2.41E-08
Lymphocyte activation	9	9.7%	3.54E-08
Inflammatory response	9	9.7%	5.31E-08
Lymphocyte proliferation	7	7.5%	5.35E-08
Response to stress	17	18.3%	3.60E-07
Chemotaxis	7	7.5%	1.17E-06
Taxis	7	7.5%	1.17E-06

a. Gene expression profiles of DSS treatment group was compared to that of *H. bilis* + DSS group and differentially expressed genes were filtered by false discovery rate $q \leq 0.05$ and estimated fold change ≥ 2
b. The functional overrepresentation of differentially expressed genes was analyzed by DAVID bioinformatics resource 2007. Fisher exact tests were performed to calculate *P* values and determine which particular annotation categories were over-represented.

Table 2. The representative mucosal differentially expressed genes derived from a comparison between DSS group and *H. bilis* + DSS group.

Gene Name[a]	GeneBank ID	Fold change[b]	*q* values	Annotation[c]
Cd28: CD28 antigen	NM_007642	5.4	0.016	Immune cell activation
Fcgr2b: Fc receptor, IgG, low affinity IIb	BM224327	2.4	0.028	Immune cell activation
Fyb: FYN binding protein	BB157866	2.3	0.050	Immune cell activation
Itagl: integrin alpha L (Cd11a)	BI554446	3.6	0.039	Immune cell activation
Irf4: interferon regulatory factor 4	U34307	6.1	0.010	Immune cell activation
Lilrb3:leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3	U96693	3.0	0.035	Immune cell activation
Lst1: leukocyte specific transcript 1	U72644	2.2	0.035	Immune cell activation
Lat2: linker for activation of T cells family, member 2	AF257136	2.8	0.004	Immune cell activation
Lcp2: lymphocyte cytosolic protein 2	BC006948	2.4	0.049	Immune cell activation
Tnfsf13b: tumor necrosis factor receptor superfamily, member 13b	AK004668	6.5	0.035	Immune cell activation
Itgam: integrin alpha M (Cd11b)	NM_008401	2.7	0.044	Immune cell activation /Chemotaxis
Itgb2: integrin beta 2 (Cd18)	NM_008404	3.1	0.032	Immune cell activation /Chemotaxis
Ccl8: chemokine (C-C motif) ligand 8	NM_021443	7.0	0.037	Chemotaxis
Ccr10: chemokine (C-C motif) receptor 10	AF215982	3.4	0.004	Chemotaxis
Ccr5: chemokine (C-C motif) receptor 5	X94151	3.5	0.047	Chemotaxis
C3ar1: complement component 3a receptor 1	NM_009779	2.1	0.049	Chemotaxis
Fcer1g: Fc receptor, IgE, high affinity I, gamma polypeptide	NM_010185	2.3	0.032	Chemotaxis
Vcam1: vascular cell adhesion molecule 1	BB250384	3.5	0.044	Chemotaxis

a. Gene expression profiles of DSS treatment group was compared to that of *H. bilis* + DSS group and differentially expressed genes were filtered by false discovery rate $q \leq 0.05$ and estimated fold change ≥ 2.

b. Fold change was defined as the comparative gene expression levels between *H. bilis* + DSS group and DSS alone group.

c. The functional overrepresentation of differentially expressed genes was analyzed by DAVID bioinformatics resource 2007.

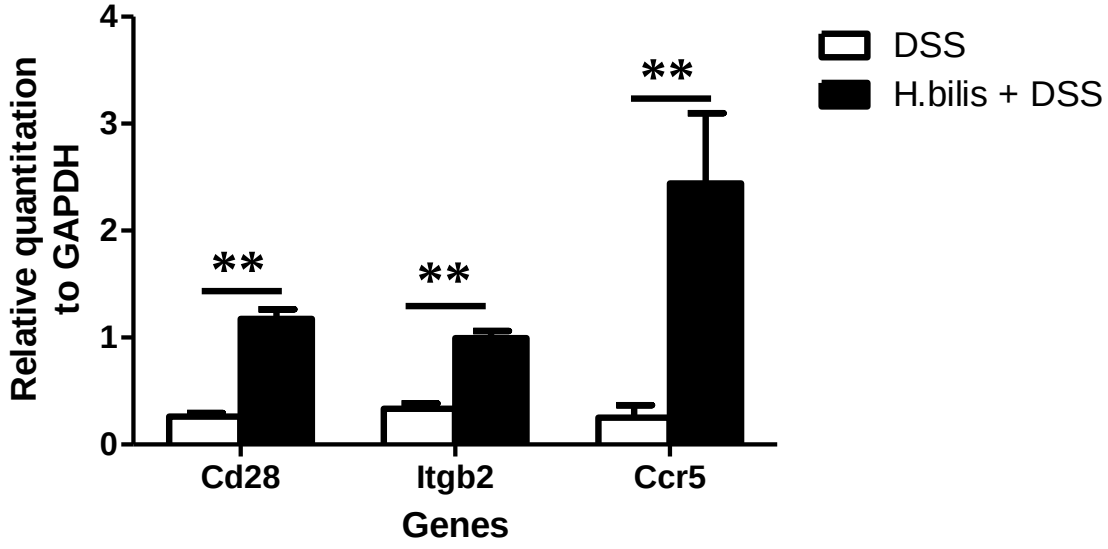


Figure 2. Analysis of mRNA expression of selected genes in the colon of mice treated with DSS alone or treated with *H. bilis* plus DSS. Gnotobiotic mice had been colonized with *H. bilis* for six weeks prior to exposure to 1.5 % DSS in their drinking water. The mice were exposed to DSS for five days followed by a four day restitution period. At this time, total RNA was isolated from colonic tissues and converted into cDNA. cDNA were then amplified via quantitative PCR using gene specific primers. The gene expression levels were normalized against the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). *Cd28*: CD28 antigen; *Itgb2*: Integrin beta 2; *Ccr5*: chemokine receptor 5. Open bars represent DSS group, filled bars represent *H. bilis* + DSS group. n = 6-8 mice per group. ** $P<0.01$.

Helicobacter bilis colonization increased the infiltration/proliferation of mucosal and/or MLN macrophages, T cells and B cells and but not neutrophils. To dissect the cellular components of mucosa after *H. bilis* colonization and/or DSS treatment, we analyzed the mucosal neutrophils, macrophages, T cells, and B cells. Neutrophil infiltration is a prominent feature of acute inflammation. MPO is an enzyme located in neutrophil granules and is an indictor of neutrophil or granulocyte infiltration [39]. We measured the MPO levels after DSS treatment and/or *H. bilis* colonization by normalizing against total protein. The *H. bilis* plus DSS treated group had significantly lower MPO levels ($P<0.05$) compared to the DSS alone group (Fig. 3). In addition, increased neutrophil infiltration in histological sections in *H. bilis* plus DSS group was not observed (data not shown). This result suggested that neutrophil infiltration might not play a major role in the development of inflammation induced by exposure to low dose DSS in gnotobiotic mice previously colonized with *H. bilis*.

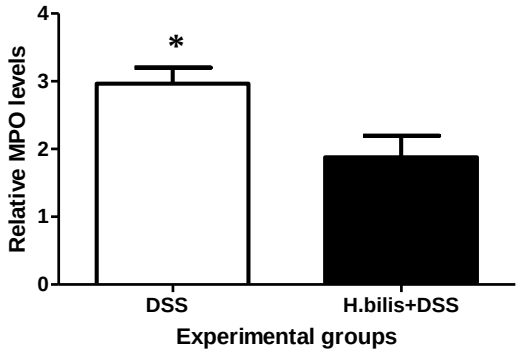


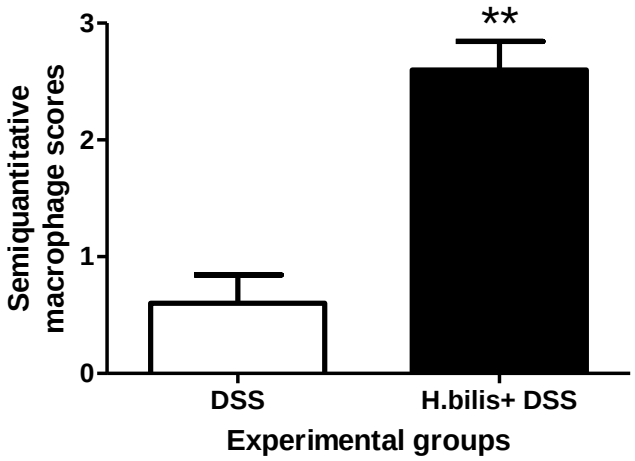
Figure 3. Analysis of colonic myeloperoxidase (MPO) levels in the colon of mice treated with DSS alone or treated with *H. bilis* plus DSS. Gnotobiotic mice had been colonized with *H. bilis* for six weeks prior to exposure to 1.5 % DSS in their drinking water. The mice were exposed to DSS for five days followed by a four day restitution period. At this time, colonic tissue samples were homogenized and sonicated. The resulting lysate were centrifuged and MPO levels in supernatants were measured as described in Materials and Methods. Open bars represent DSS group, filled bars represent *H. bilis* + DSS group. n = 10 to 14 mice per group. * $P < 0.05$ vs. *H. bilis* + DSS group.

Tissues were also prepared for immunohistochemical analysis to detect the presence of macrophages using the F4/80 monoclonal antibody. Microscopic analysis revealed a notable increase in the numbers of macrophages in the lamina propria and submucosa of mice in the *H. bilis* + DSS group, in comparison to mice treated with DSS alone (Fig. 4). Interestingly, macrophages were observed within ulcerative lesions of mice in the *H. bilis* + DSS group, indicating the macrophage infiltration may be involved in the development of inflammatory lesions (Fig. 4).

In order to study the effects of *H. bilis* on mucosal and MLN T and B cells, LPL and MLN cells were isolated and single cell suspensions were prepared. Based upon flow cytometric analysis, the results showed that LPL populations from mice in the *H. bilis* + DSS group had a significant ($P < 0.05$) increase of approximately 2-fold in total $CD3^+$ T cells and a moderate increase in total $CD19^+$ B cells in the intestinal mucosa, in comparison to cells recovered from mice treated with DSS alone (Fig. 5A). With regards to MLN cells, *H. bilis* colonization induced a significant ($P < 0.01$) increase in the total numbers of both $CD3^+$ T cells and $CD19^+$ B cells (Fig. 5B). While total numbers of $CD3^+$ T cells and $CD19^+$ B cells in the mucosa were similiar, the number of $CD3^+$ T cells were much higher than those of $CD19^+$cells in MLNs of mice colonized with *H. bilis*. Consistent with the increased numbers of T cells and B cells in MLN, there was a significant increase in antigen-specific proliferative responses of MLN cells upon the stimulation with APC pulsed with ASF or *H. bilis* whole cell lysates (Fig. 6). These results demonstrate the presence of antigen-specific lymphocytes in the MLN of mice following *H. bilis* colonization.

Consistent with the activation and proliferation of B cells, a strong increase in antibody responses to both *H. bilis* and commensal bacteria ASF was observed in serum samples obtained from mice colonized with *H. bilis* and treated with DSS, in comparison to mice treated with DSS alone (Fig. 7A, B, C). Notably, IgG_{2a} antibody responses predominated over IgG_1 antibody responses to both *H. bilis* and ASF (Fig. 7A, B, C). In addition, IgG_{2a} antibody responses to some commensal bacteria such as ASF 356, 502, and 519 in mice from the *H. bilis* + DSS group were higher than responses to other ASF antigens (Fig. 7C).

(A)


**
3
2
1
0
Semiquantitative macrophage scores
DSS
H.bilis+ DSS
Experimental groups

(B)

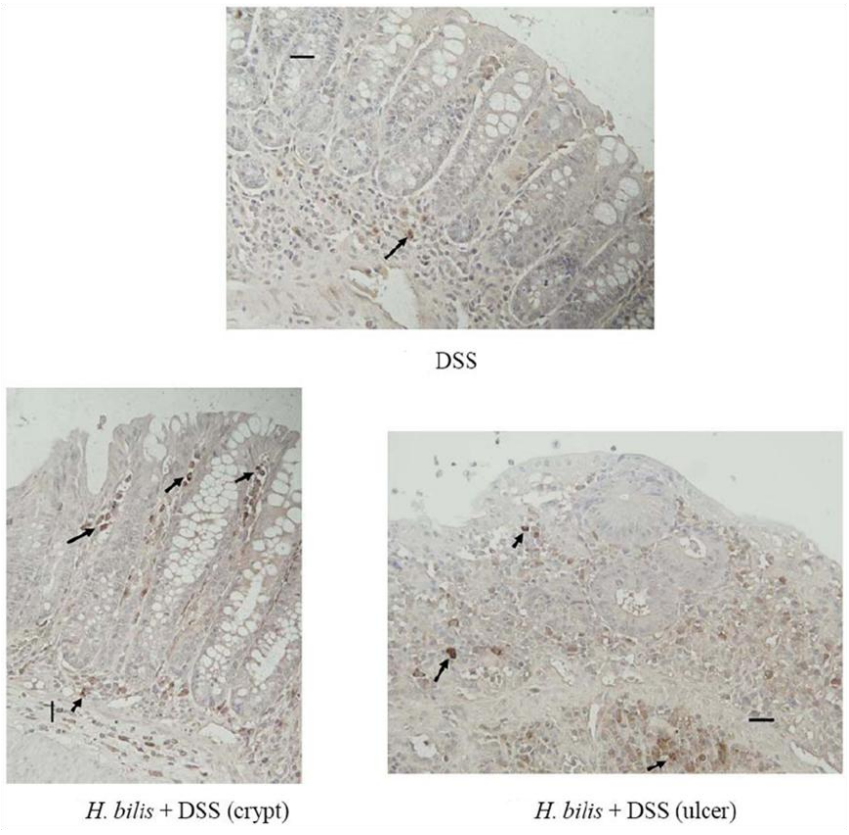
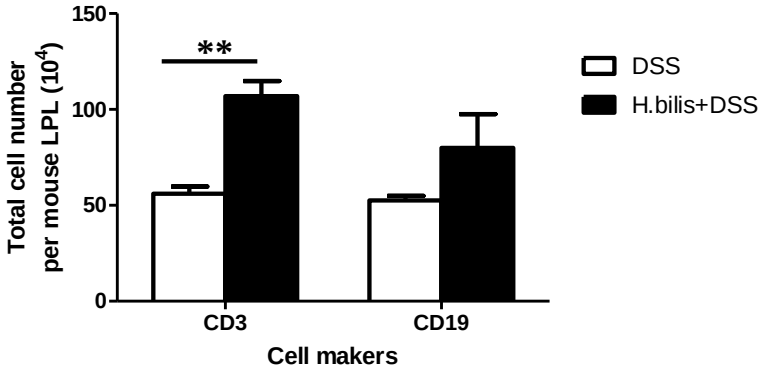

DSS
H. bilis + DSS (crypt)
H. bilis + DSS (ulcer)

Figure 4. Immunohistochemical analysis of F4/80$^+$ macrophages in colonic mucosa of mice treated with DSS alone or treated with *H. bilis* plus DSS. Gnotobiotic mice had been colonized with *H. bilis* for six weeks prior to exposure to 1.5 % DSS in their drinking water. The mice were exposed to DSS for five days followed by a four day restitution period. At this time, colonic tissues were fixed and routinely processed as described in Materials and Methods. Paraffin-embedded colon tissue sections were deparaffinized, rehydrated and treated with protease enzymatic digestion. The sections were then stained with rat anti-mouse F4/80 monoclonal antibody followed by biotin labeled goatanti-rat secondary antibody and HRP-Streptavidin. The sections were incubated with a diaminobenzidine substrate to reveal brown/black staining for F4/80$^+$ cells. Black bars represent 50 μm. The arrows point to representative F4/80$^+$ cells. (A) Semiquantitative analysis of F4/80$^+$ cells (B) Representative photomicrographs of F480$^+$ cells. 40 x magnifications. n =5-6 mice. P<0.01.

***Helicobacter bilis* colonization downregulated the expression levels of detoxification-associated genes**. Intestinal epithelial cells participate in the process of detoxification and biotransformation of drugs and xenobiotics [40]. Loss or dysregulation of detoxification and biotransformation processes by the intestinal epithelium may contribute to the initiation and progression of IBD [41]. In this regard, *H. bilis* colonization was shown to induce the downregulation of detoxification-associated genes such as *Cyp4b1* (Cytochrome P450, family 4, subfamily b, polypeptide 1) and *Ugt8a* (UDP galactosyltransferase 8A). The downregulation of these genes might create a predisposition of the host to the development of colitis (Chapter 2).

In order to further examine the effects of *H. bilis* on DSS-induced colitis, the expression levels of some detoxification-associated genes in intestine mucosa were analyzed. The results showed that mice in the *H. bilis* + DSS group had significantly (P < 0.05) lower expression levels of mRNA for genes associated with phase I (i.e., *Cyp4b1*) and phase II (i.e., *Ugt8a*) detoxification/biotransformation than mice treated with DSS alone (Fig. 8). These results indicate that *H. bilis* colonization affected the detoxification function of the intestinal epithelium and potentially contributed to the increased susceptibility of these mice to DSS-induced colitis.

(A)



(B)

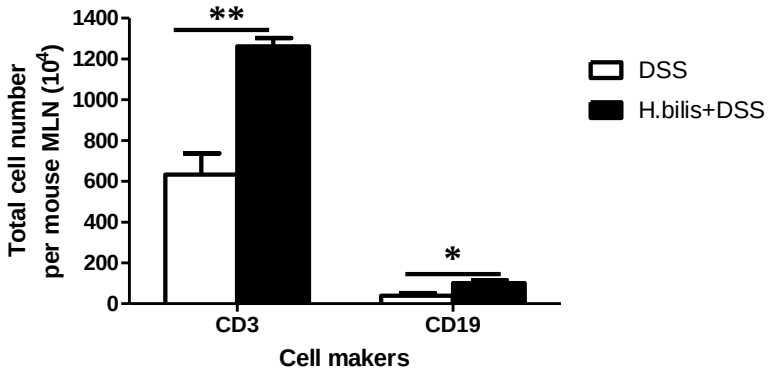


Figure 5. Effects of *Helicobacter bilis* colonization on the total $CD3^+$ T cell and $CD19^+$ B cells in lamina propria and mesenteric lymph node (MLN) following exposure to DSS. At necropsy, mouse lamina propria lymphocytes (LPL) and MLN cells were isolated and total cell numbers were counted. The percentage of $CD3^+$ and $CD19^+$ cells in total cells were measured by flow cytometry. The data presented represent the total $CD3^+$ and $CD19^+$ cells per mouse colon or MLN. (A) Total numbers of $CD3^+$ and $CD19^+$ LPL (B) Total numbers of $CD3^+$ and $CD19^+$ MLN cells. Open bars represent DSS group, filled bars represent *H. bilis* + DSS group. n = 6-8 mice. *$P < 0.05$, ** $P < 0.01$.

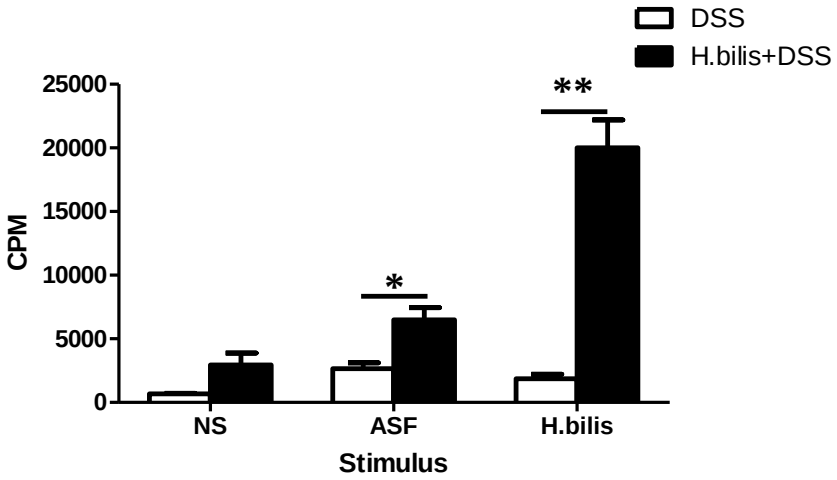
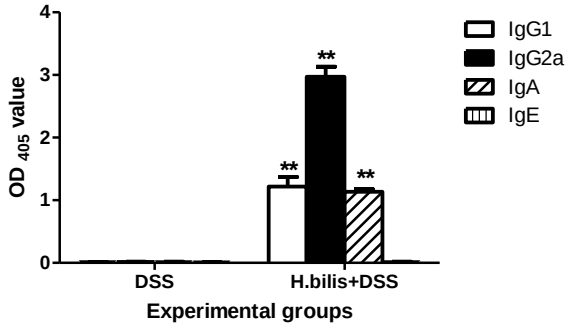
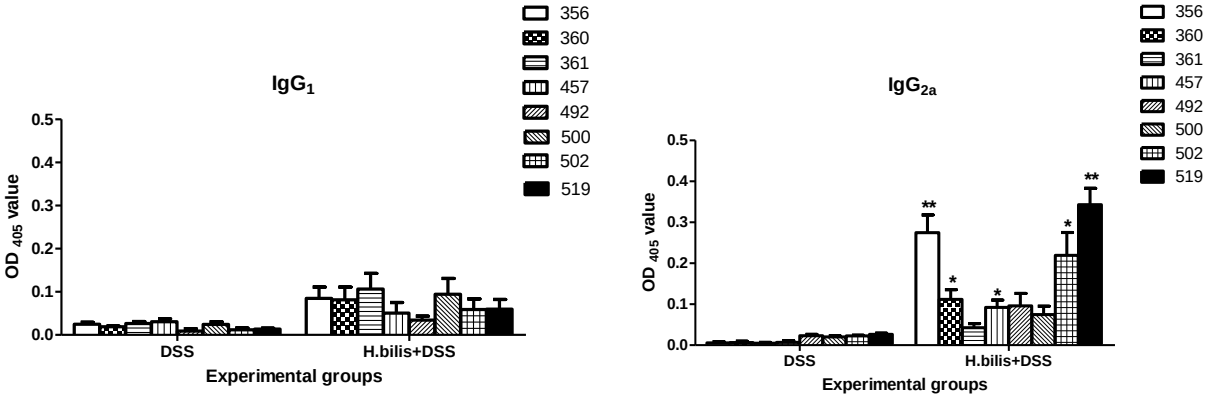


Figure 6. Analysis of antigen-specific mesenteric lymph node (MLN) cell proliferation of cell recovered from mice treated with DSS alone or treated with *H. bilis* plus DSS. Gnotobiotic mice had been colonized with *H. bilis* for six weeks prior to exposure to 1.5 % DSS in their drinking water. The mice were exposed to DSS for five days followed by a four day restitution period. At this time, MLN cells and LPLs were recovered from individual mice. 2×10^6 cells/ml unfractioned MLN cells were incubated for 5 days with antigen-presenting cells (1:1) that had been incubated overnight with or without whole cell lysates of the altered Schaedler's flora organisms, *H. bilis*. ^{3}H-Thymidine was added into cell supernatant 8 hours before harvest and proliferation was measured by liquid scintillation. Open bars represent DSS alone group, filled bars represent *H. bilis* + DSS group. n = 6-8 mice per group. *$P < 0.05$, ** $P < 0.01$.



(A) Antibody responses to *Helicobacter bilis*



(B) IgG1 antibody responses to ASF

(C) IgG2a antibody responses to ASF

Figure 7. Measurement of antibody responses to *Helicobacter bilis* and altered Schadler's flora (ASF) in the sera of mice treated with DSS alone or treated with *H. bilis* + DSS. Gnotobiotic mice had been colonized with *H. bilis* for six weeks prior to exposure to 1.5 % DSS in their drinking water. The mice were exposed to DSS for five days followed by a four day restitution period. Sera samples collected at necropsy were diluted and added into the 96-well microtiter plates coated with the whole cell lysates of *H. bilis* or each ASF. Isotype specific antibody responses were measured as described in Materials and Methods. n = 6 to 10 mice. * P<0.05, ** P<0.01 vs. DSS group. (A) Antibody responses to *H. bilis.* (B) IgG_1 to ASF. (C) IgG_{2a} to ASF.

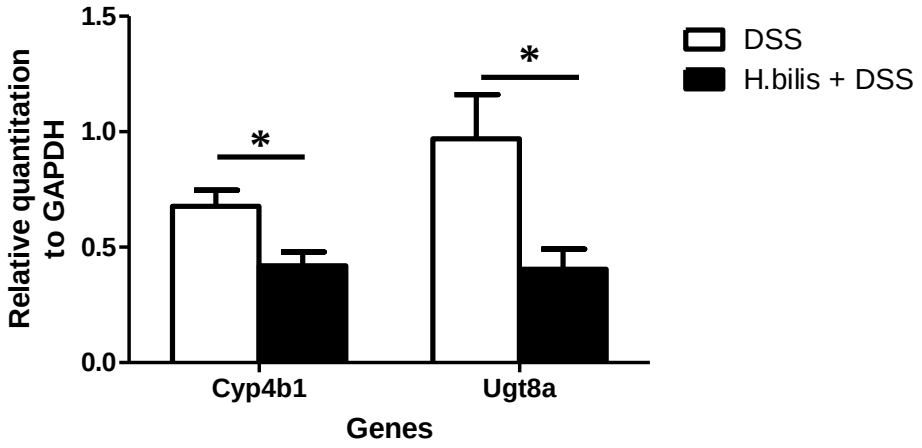


Figure 8. Analysis of mRNA expression for detoxification-associated genes in the colon of mice treated with DSS alone or treated with *H. bilis* plus DSS. Gnotobiotic mice had been colonized with *H. bilis* for six weeks prior to exposure to 1.5 % DSS in their drinking water. The mice were exposed to DSS for five days followed by a four day restitution period. Total RNA was isolated from colonic tissues and converted into cDNA. cDNA samples were then amplified via quantitative PCR using gene specific primers. The mRNA expression levels were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH). *Cyp4b1*: Cytochrome P450, family 4, subfamily b, polypeptide 1. *Ugt8a*: UDP galactosyltransferase 8A. Open bars represent DSS group, filled bars represent *H. bilis* + DSS group. n = 6 to 8 mice per group. * $P<0.05$.

DISCUSSION

In the present study, it was shown that *Helicobacter bilis* colonization exacerbated colitis in immunocompetent gnotobiotic mice. *H. bilis* colonized mice developed more severe colitis following exposure to a low dose of DSS compared to non-*H. bilis* colonized control mice. This increased sensitivity to a colitic insult was accompanied by an increase in the colonic expression mRNA specific for adhesion molecules and chemokines/chemokine receptors, such as *Itgb2, Ccl8, Ccr5*. Cellular analysis also indicated that *H, bilis* colonization increased the infiltration of leukocytes including macrophages, T cells, B cells into the lamina propria of mice treated with DSS. More importantly, the infiltrated leukocytes appeared to be

activated as suggested by the upregulation of mRNA specific for T cell and B cell activation markers (e.g., *Cd28, Tnfsf13b)* and increased antigen-specific antibody responses to both *H. bilis* and commensal ASF bacteria. These results suggested that colonization of mice with *H. bilis* has a potent immunomodulatory effect that provides a predisposing stimulus towards the development of colitis. While playing the role of provocateur, *H. bilis* colonization alone does not induce overt-inflammation in an immunocompetent host [18]. The results of the present study supports the contention that *H. bilis*, at least in part, through the recruitment and/or activation of mucosal immune cells, is capable of priming a mucosal immune response that contributes to initiation and/or exacerbation of IBD. *Helicobacter bilis* has been shown to be prevalent in many rodent colonies used for biomedical research and this has confounded the analysis of many studies [8, 12]. Because *H. bilis* has been also shown to be present in the human gut [9, 10], the elucidation of deleterious effects of *H. bilis* colonization has upon the development of colitis has the potential to improve biomedical research and enhance treatment of IBD patients.

In contrast to increased histological lesions and upregulation of some adhesion molecules potentially involved in neutrophil infiltration (e.g., *Itgb2/Cd18*), mice treated with *H. bilis* plus DSS had significantly lower colonic myeloperoxidase levels than mice treated with DSS alone. Histological examination also showed that the expected infiltration of neutrophils into the colonic tissues of mice treated with *H. bilis* plus DSS did not occur. The lack of a demonstrable neutrophil infiltration suggested that; 1) neutrophils might infiltrate into the colonic mucosa at an earlier time point (e.g., prior to the DSS restitution period), or 2) prior *H. bilis* colonization facilitated the induction of chemokine responses that favored recruitment of other immune cells, or 3) *H. bilis* itself might have some pathogenic factors that inhibit the infiltration or activation or neutrophils. Regardless, the recruitment/activation of macrophages and lymphocytes induced by *H. bilis* colonization indicates that any neutrophilic infiltration that did occur was not sufficiently effective to prevent immuno-stimulatory effects of *H. bilis*. Defective neutrophil functions and the presence of lamina propria graulomas are frequently observed in patients with Crohn's disease [42-44]. In this context, the present results indicate that intestinal inflammation induced by DSS treatment in *H. bilis* colonized mice appears to mimic the characteristic lesions of Crohn's disease rather

than ulcerative colitis. Also, the antibody responses to both *H. bilis* and ASF suggest a Th1-bias (IgG_{2a} vs. IgG_1) which is similar to that observed in patients with Crohn's disease [6].

Macrophages have been implicated to be a crucial cell type involved in the pathogenesis of DSS-induced colitis [45, 46]. The present study demonstrated that *H. bilis* colonization increased the infiltration of $F4/80^+$ matures macrophages into lamina propria and submucosa of mice treated with DSS. In addition, the upregulation of mRNA expression for genes associated with macrophage cell surface molecules (i.e., *Igtam/Cd11b*) and chemokines (e.g., *Ccl8*) was observed in tissues from mice with DSS-induced colitis. As these results do not elucidate a cause or effect, further investigations are needed to clearly define the role of macrophages in the lamina propria of mice colonized by *H. bilis*.

The activation and recruitment of lymphocytes into the lamina propria indicated that *H. bilis* colonization induced the activation of adaptive immunity. Notably, *H. bilis* colonization induced host antibody responses to commensal bacteria ASF356, 502, and 519, as well as antibody responses to *H. bilis*. Immune responses to commensal bacteria have been suggested to be one of the key mechanisms associated with the development of colitis [6]. Furthermore, ASF 356, 502, and 519 are members of the *Clostridium* or *Bacteroides* species that have been shown to exacerbate the disease activity in IBD patients [47, 48].

Of particular importance, the downregulation of mRNA specific for two detoxification-associated genes was observed in mucosa of mice colonized with *H. bilis* and treated with DSS. Dysregulation of detoxification genes of intestinal epithelial cells has been implicated in IBD patients and animal models [41].This result indicated that *H. bilis* colonization might affect the detoxification ability and barrier function of intestinal epithelial cells. Impaired epithelial functions, such as increased permeability, have been shown to be the primary defect and lead to activation of adaptive immunity and development of intestinal inflammation in SAMP1/Yit mice [49]. It would be of considerable interest to determine whether alteration of potential epithelial functions (e.g., detoxification activity) can initiate or perpetuate the development of adaptive immunity including recruitment/activation of mucosal immune cells that results in chronic mucosal inflammation.

Despite the potential effects on detoxification activity and recruitment/activation of mucosal immune cells, *H. bilis* colonization alone did not induce overt mucosal inflammation.

The requirement of both *H. bilis* colonization and a second colitic insult (e.g., low-dose DSS) in order to induce the development of severe colitis is consistent with the "multiple-hit hypothesis" for the development of IBD [4]. It was suspected that each factor alone (e.g., *H. bilis* or DSS) plays distinct roles in altering the mucosal functions and the combination of these two factors leads to the development of colitis. The effects of *H. bilis* seem to alter the epithelial barrier function and recruit/activate the resident immune cells in lamina propria establishing a state of subclinical inflammation. The effects of DSS treatment appear to change the epithelial barrier function by reducing the colonic epithelial proliferation leading to loss of the progenitor cell compartment [50] and exposing the underlying lamina propria to a multitude of phlogistic components and antigens. Indeed, there was an observed downregulation of genes associated with the cellular proliferation following DSS treatment (data not shown). Subsequently, the deterioration of barrier function [51] allows gut flora, including *H. bilis,* to further activate the mucosal innate immune cell population, resulting in chronic, mucosal inflammation [52].

In summary, *Helicobacter bilis* increased the host sensitivity to DSS-induced colitis in immunocompetent mice, probably due to the increased recruitment and/or activation of intestinal inflammatory cells such as macrophage, T cells, and B cells in combination with the dysregulation of epithelial detoxification processes. The results of the present studies suggest that persistent colonization by bacterial provocateurs such as *Helicobacter bilis* may be a potential predisposing environmental factor in the initiation and/or exacerbation of colitis.

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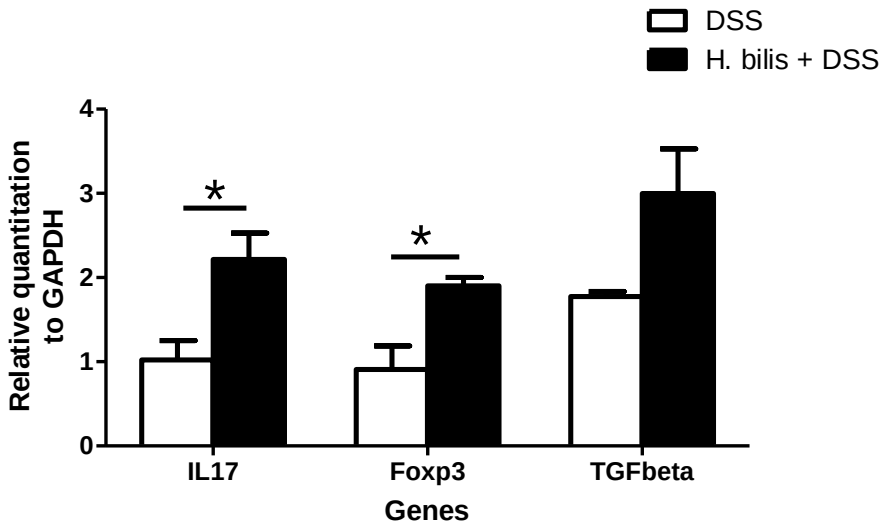

Supplemental Figure 1. Analaysis of mRNA of selected genes associated with lamina prorprial lymphocytes (LPL) recovered from mice treated with DSS alone or treated with *H. bilis* plus DSS. Gnotobiotic mice had been colonized with *H. bilis* for six weeks prior to exposure to 1.5 % DSS in their drinking water. The mice were exposed to DSS for five days followed by a four day restitution period. At this time, total RNA was isolated from colonic tissues and converted into cDNA. cDNA were then amplified via quantitative PCR using gene specific primers. The gene expression levels were normalized into glyceraldehyde-3-phosphate dehydrogenase (GAPDH). *IL17*: Interleukin 17, *Foxp3*: Forkhead box P3, *TGFβ*: Transforming growth factor, beta. Open bars represent DSS group, filled bars represent *H. bilis* + DSS group. n = 6-8 mice per group. * $P<0.05$.

Supplemental Table 1. The list of mucosal differentially expressed genes derived from a comparison between *H. bilis* + DSS group and DSS group.

Gene[a]	Gene Title	Gene Symbol	Fold change[b]	GeneBank ID	*q* values
1452565_x_at	---	---	5.0	M11024	0.036
1435560_at	---	---	3.6	BI554446	0.039
1421420_at	---	---	3.4	AF215982	0.004
1448061_at	---	---	3.3	AA183642	0.029
1425471_x_at	---	---	2.5	BC003855	0.033
1418204_s_at	allograft inflammatory factor 1	Aif1	2.6	NM_019467	0.039
1452535_at	Anti-CD4 immunoglobulin heavy chain (VH2-DQ52-JH3 region)	---	4.8	Z95476	0.035
1417597_at	CD28 antigen	Cd28	5.4	NM_007642	0.016
1437025_at	CD28 antigen	Cd28	2.9	AV313615	0.028
1427301_at	CD48 antigen	Cd48	3.2	BE634960	0.035
1426112_a_at	CD72 antigen	Cd72	4.2	BC003824	0.044
1436530_at	CDNA clone MGC:107680 IMAGE:6766535	---	2.2	AA666504	0.035
1419684_at	chemokine (C-C motif) ligand 8	Ccl8	7.0	NM_021443	0.037
1422259_a_at	chemokine (C-C motif) receptor 5	Ccr5	3.5	X94151	0.047
1417381_at	complement component 1, q subcomponent, alpha polypeptide	C1qa	2.3	NM_007572	0.042
1417063_at	complement component 1, q subcomponent, beta polypeptide	C1qb	2.7	NM_009777	0.039
1434366_x_at	complement component 1, q subcomponent, beta polypeptide	C1qb	2.2	AW227993	0.039
1449401_at	complement component 1, q subcomponent, gamma polypeptide	C1qg	2.4	NM_007574	0.044
1442082_at	complement component 3a receptor 1	C3ar1	2.9	BB333624	0.032
1419483_at	complement component 3a receptor 1	C3ar1	2.6	NM_009779	0.035
1419482_at	complement component 3a receptor 1	C3ar1	2.1	NM_009779	0.049
1425407_s_at	C-type lectin domain family 4, member a2	Clec4a2	2.1	BC006623	0.005
1429954_at	C-type lectin domain family 4, member a3	Clec4a3	4.8	AK014135	0.006
1425951_a_at	C-type lectin domain family 4, member n	Clec4n	2.7	AF240358	0.049
1419627_s_at	C-type lectin domain family 4, member n	Clec4n	2.5	NM_020001	0.032
1422978_at	cytochrome b-245, beta polypeptide	Cybb	3.1	NM_007807	0.022
1436778_at	cytochrome b-245, beta polypeptide	Cybb	3.0	AV373944	0.022
1436779_at	cytochrome b-245, beta polypeptide	Cybb	2.9	AV373944	0.036
1435343_at	dedicator of cytokinesis 10	Dock10	2.2	BF715043	0.035
1453677_a_at	Der1-like domain family, member 3	Derl3	3.5	AK007348	0.006
1450241_a_at	ecotropic viral integration site 2a	Evi2a	2.8	NM_010161	0.049
1451161_a_at	EGF-like module containing, mucin-like, hormone receptor-like sequence 1	Emr1	2.1	U66888	0.039
1422120_at	ELL associated factor 2	Eaf2	4.1	AY034479	0.049
1445688_at	Endothelin converting enzyme 1 (Ece1), mRNA	Ece1	2.0	BG073119	0.036
1434067_at	expressed sequence AI662270	AI662270	5.7	BE688410	0.022
1434068_s_at	expressed sequence AI662270	AI662270	4.4	BE688410	0.038
1418340_at	Fc receptor, IgE, high affinity I, gamma polypeptide	Fcer1g	2.3	NM_010185	0.032
1417876_at	Fc receptor, IgG, high affinity I	Fcgr1	2.4	AF143181	0.032
1435477_s_at	Fc receptor, IgG, low affinity IIb	Fcgr2b	2.4	BM224327	0.028

Supplemental Table 1 (Continued)

Gene[a]	Gene Title	Gene Symbol	Fold change[b]	GeneBank ID	*q* values
1455332_x_at	Fc receptor, IgG, low affinity IIb	Fcgr2b	2.1	BM224327	0.014
1451941_a_at	Fc receptor, IgG, low affinity IIb	Fcgr2b	2.0	M14216	0.032
1422953_at	formyl peptide receptor, related sequence 2	Fpr-rs2	2.8	NM_008039	0.049
1452117_a_at	FYN binding protein	Fyb	2.3	BB157866	0.050
1431296_at	G protein-coupled receptor 15	Gpr15	2.1	AA555873	0.049
1424927_at	GLI pathogenesis-related 1 (glioma)	Glipr1	2.5	BC025083	0.026
1420394_s_at	glycoprotein 49 A	Gp49a	2.5	U05264	0.028
1449175_at	G-protein coupled receptor 65	Gpr65	2.2	NM_008152	0.049
1446745_at	hypothetical LOC433637	LOC433637	2.2	BB490675	0.021
1460423_x_at	Ig kappa chain	IgM	48.1	U29768	0.011
1427851_x_at	immunoglobulin heavy chain (J558 family)	Igh-VJ558	29.8	AF065324	0.011
1429381_x_at	immunoglobulin heavy chain (J558 family)	Igh-VJ558	3.1	AK007826	0.006
1425763_x_at	immunoglobulin heavy chain (J558 family)	Igh-VJ558	2.4	BC019425	0.013
1421653_a_at	immunoglobulin heavy chain (J558 family)	Igh-VJ558	2.1	NM_134051	0.003
1424305_at	immunoglobulin joining chain	Igj	4.4	BC006026	0.004
1427757_at	immunoglobulin kappa chain variable 21 (V21)	Igk-V21	31.7	D29670	0.049
1452417_x_at	immunoglobulin kappa chain, constant region	Igk-C	2.3	AV057155	0.007
1427455_x_at	immunoglobulin kappa chain, constant region	Igk-C	2.3	BI107286	0.011
1427660_x_at	immunoglobulin kappa chain, constant region	Igk-C	2.1	BC013496	0.006
1452536_s_at	Immunoglobulin kappa light variable region (IgKV gene)	Igk-V1	29.4	Z95478	0.011
1427858_at	Immunoglobulin kappa light variable region (IgKV gene)	Igk-V1	3.5	U29768	0.022
1452463_x_at	Immunoglobulin lambda chain, mAb 667	Igk-V8	3.6	BG966217	0.042
1426200_at	Immunoglobulin lambda chain, mAb 667	Igk-V8	3.4	AY058910	0.044
1451951_at	Immunoglobulin light chain variable region (IGLV gene), clone F22-4	Igk-V8	27.1	U62386	0.011
1421408_at	immunoglobulin superfamily, member 6	Igsf6	2.1	NM_030691	0.044
1427262_at	inactive X specific transcripts	Xist	1159.1	L04961	0.000
1436936_s_at	inactive X specific transcripts	Xist	122.6	BG806300	0.006
1422046_at	integrin alpha M	Itgam	2.7	NM_008401	0.044
1450678_at	integrin beta 2	Itgb2	3.1	NM_008404	0.032
1421173_at	interferon regulatory factor 4	Irf4	6.1	U34307	0.010
1423555_a_at	interferon-induced protein 44	Ifi44	8.2	BB329808	0.044
1416296_at	interleukin 2 receptor, gamma chain	Il2rg	2.8	L20048	0.042
1424302_at	leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3	Lilrb3	3.0	U96693	0.035
1425548_a_at	leukocyte specific transcript 1	Lst1	2.2	U72644	0.035
1426169_a_at	linker for activation of T cells family, member 2	Lat2	2.8	AF257136	0.004
1421571_a_at	lymphocyte antigen 6 complex, locus C	Ly6c	3.6	NM_010741	0.044
1418641_at	lymphocyte cytosolic protein 2	Lcp2	2.4	BC006948	0.049
1427076_at	macrophage expressed gene 1	Mpeg1	2.1	L20315	0.028
1422062_at	macrophage scavenger receptor 1	Msr1	2.0	BC003814	0.035

Supplemental Table 1 (Continued)

Gene[a]	Gene Title	Gene Symbol	Fold change[b]	GeneBank ID	*q* values
1419599_s_at	membrane-spanning 4-domains, subfamily A, member 11	Ms4a11	4.5	NM_026835	0.022
1419598_at	membrane-spanning 4-domains, subfamily A, member 6D	Ms4a6d	4.2	NM_026835	0.022
1424754_at	membrane-spanning 4-domains, subfamily A, member 7	Ms4a7	2.0	BC024402	0.039
1427577_x_at	Monoclonal antiidiotypic antibody IgK (hypervariable region) mRNA	---	8.3	BF301241	0.010
1427576_at	Monoclonal antiidiotypic antibody IgK (hypervariable region) mRNA	---	6.0	BF301241	0.022
1431320_a_at	myosin Va	Myo5a	2.3	AK002362	0.045
1428786_at	NCK associated protein 1 like	Nckap1l	2.0	BM238906	0.028
1418465_at	neutrophil cytosolic factor 4	Ncf4	2.5	NM_008677	0.039
1451821_a_at	nuclear antigen Sp100	Sp100	2.8	U83636	0.044
1417483_at	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	Nfkbiz	2.7	AB026551	0.044
1430700_a_at	phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)	Pla2g7	2.2	AK005158	0.035
1417426_at	proteoglycan 1, secretory granule	Prg1	2.8	NM_011157	0.044
1428947_at	RIKEN cDNA 2010001M09 gene	2010001M09Rik	19.2	AK008016	0.011
1424704_at	runt related transcription factor 2	Runx2	2.5	D14636	0.049
1450165_at	schlafen 2	Slfn2	2.1	NM_011408	0.049
1425738_at	similar to anti-glycoprotein-B of human Cytomegalovirus immunoglobulin Vl chain	LOC243469	40.2	M35669	0.006
1453472_a_at	SLAM family member 7	Slamf7	2.2	AK016183	0.042
1420819_at	src-like adaptor	Sla	2.1	NM_009192	0.050
1419186_a_at	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 4	St8sia4	2.4	NM_009183	0.044
1456147_at	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 6	st8sia6	3.7	BB486599	0.018
1457753_at	toll-like receptor 13	Tlr13	2.4	BI655907	0.044
1448021_at	Transcribed locus	---	5.5	AA266723	0.039
1435144_at	Transcribed locus	---	2.8	BM243379	0.014
1451860_a_at	tripartite motif protein 30	Trim30	3.2	AF220015	0.049
1423182_at	tumor necrosis factor receptor superfamily, member 13b	Tnfrsf13b	6.5	AK004668	0.035
1420782_at	tumor necrosis factor receptor superfamily, member 17	Tnfrsf17	6.4	NM_011608	0.029
1446234_at	Ubiquitously transcribed tetratricopeptide repeat gene , X chromosome, mRNA (cDNA clone IMAGE:5356391)	Utx	2.5	BB125985	0.049
1448162_at	vascular cell adhesion molecule 1	Vcam1	3.4	BB250384	0.044

a. Gene expression profiles of DSS treatment group was compared to that of *H. bilis* + DSS group and differentially expressed genes were filtered by false discovery rate $q \leq 0.05$ and estimated fold change ≥ 2.

b. Fold change was defined as the comparative gene expression levels between *H. bilis* + DSS group and DSS alone group.

CHAPTER 4: ORAL TREATMENT WITH HYPOXOSIDE AMELIORATES *Brachyspira hyodysenteriae*-INDUCED MURINE COLITIS

A paper to be submitted to the journal *American Journal of Physiology - Gastrointestinal and Liver Physiology*

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ABSTRACT

Brachyspira hyodysenteriae is the causative agent of swine dysentery and induces a characteristic mucosal inflammatory response resulting in pronounced typhlocolitis in mice and swine. Hypoxoside has been reported to be an anti-inflammatory drug extracted from an African medicinal plant, *Hypoxis hemerocallidea. In vitro* studies have shown that hypoxoside inhibits the secretion of pro-inflammatory cytokines and the nuclear binding activity of NF-κB. The aim of this study was to evaluate the ability of hypoxoside to prevent or ameliorate *B. hyodysenteriae*-induced colitis. Mice were orally treated with hypoxoside for seven days prior to *B. hyodysenteriae* infection and the treatments continued daily for seven days postinfection (DPI) . At the termination of the experiment (i.e., seven DPI), gross and histological lesions, weight loss, and mucosal myeloperoxidase activity were evaluated. In addition, expression of NF-κB-associated genes, the protein level of active NF-κB subunit p65, epithelial cell proliferation, and the number of gut *B. hyodysenteriae* were also measured. Hypoxoside treatment significantly (P <0.05) reduced the severity of *B. hyodysenteriae* -induced lesions, weight loss, and tissue levels of myeloperoxidase. NF-κB-associated genes were downregulated and the protein levels of active p65, and the degree of

epithelial proliferation were decreased after hypoxoside treatment. Mechanistically, there was no direct evidence that hypoxoside was anti-microbial based on qPCR assessment of *B. hyodysenteriae* within cecal contents. In conclusion, oral treatment with hypoxoside ameliorated *B. hyodysenteriae*-induced colitis and these results suggest that pro-drugs such as hypoxoside have therapeutic potential for ameliorating enteropathies mediated by overly active host inflammatory processes.

Key Words: Hypoxoside; *Brachyspira hyodysenteriae*; colitis; NF-κB

INTRODUCTION

Inflammatory bowel diseases (IBD), including Crohn's disease and ulcerative colitis, are chronic and relapsing intestinal inflammatory disorders leading to diarrhea, abdominal pain, and weight loss. As many as 1.4 and 2.2 million individuals in the United States and Europe, respectively, are suffering from these diseases, and these diseases are reported to have a significant impact upon the cost of health care and loss of productivity (29, 46, 48). The exact etiology of IBD is still unknown though genetic defects, increased epithelial permeability, and dysregulation of host immune responses to gut flora are considered to be three major factors contributing to the pathogenesis of IBD (11, 30, 43). Mouse models of experimental colitis induced by gene knockout, chemicals, adoptive transfer, or spontaneous development are widely utilized to study the mechanisms underlying the development of IBD and to design new therapeutic strategies for the treatment of IBD (8, 10, 21). In addition, microbial infections, such as *Citrobacter rodentium, Helicobacter hepticus*, have also been used to evaluate the induction of inflammation leading to acute or chronic colitis (8, 24).

Brachyspira hyodysenteriae, a gram-negative anaerobic spirochete that is the causative agent of swine dysentery, induces colonic inflammation that is similar in both swine and mice (18, 19, 20, 36, 37, 41). In mice, the cecal histological lesions induced by *B. hyodysenteriae* include submucosal edema, inflammatory cell infiltration of the lamina propria, and erosions of mucosal epithelium (41).

Like other local inflammatory responses, *B. hyodysenteriae*-induced inflammation was associated with leukocyte infiltration, production of pro-inflammatory cytokines, and activation of NF-κB (13, 42). In comparison, excessive or inappropriate activation of NF-κB

has been also observed in human IBD and other inflammatory diseases (2, 9). In addition, blockage of NF-κB pathway serves as an important therapeutic intervention strategy for managing inflammatory diseases (2, 9).

Currently, some anti-inflammatory drugs such as 5-aminosalicylates, corticosteroids sulfasalazine are commonly used to treat IBD patients (7). However, current therapies not only show limited benefits but also have unwanted side effects (7, 16). It is, therefore, desirable to explore new classes of agents that are equally or more effective anti-inflammatory compounds and that may cause fewer deleterious side effects.

Hypoxoside is a diglucoside present in the corm of an African medicinal plant, *Hypoxis hemerocallidea* (also known as *Hypoxis rooperi,* African Potato), a member of the family *Hypoxidaceae* (1). This African traditional medicine has been widely used in southern Africa and claimed to be an effective remedy for an array of human aliments such as HIV/AIDS-related diseases, arthritis, diabetes mellitus, cancers, gastric and duodenal ulcers, and urinary tract infections (38).

In the colon, hypoxoside can be converted by β-glucosidase produced by gut bacteria into its aglucone compound called rooperol (23). It has been shown that rooperol has anti-inflammatory effects and possible usefulness in the treatment of inflammatory disease (3, 14, 15, 25, 38). For example, rooperol was shown to inhibit the *in vitro* production of TNF-α, IL-1, IL-6, and nitric oxide by endotoxin-stimulated human alveolar macrophage, human blood monocytes, histiocytic cells, and rat alveolar macrophage (14). Results of another study showed that rooperol decreased the mRNA and protein expression levels of vascular cell adhesion molecule-1(VCAM-1) and nitric oxide synthase (iNOS) in microvascular endothelial cells stimulated with TNF-α or IFN-γ (3). Rooperol was also shown to decrease the expression of mRNA specific for pro-inflammatory cytokines in human promonocytic cell line and inhibit the binding activity of transcriptional factors including NF-κB and AP-1(c-jun/c-fos dimer) (15). Recently, rooperol was shown to inhibit the enzymatic activities of cyclooxygenase-1 and 2 and might interfere with the synthesis of other inflammatory mediators such as prostaglandins (25). *In vivo,* it has been shown that oral injection of hypoxoside inhibited fresh egg albumin-induced acute inflammation (edema) within the hind paws of rats (38).

Based on these observations, it was hypothesized that orally administered hypoxoside would ameliorate mucosal inflammation through the downregulation or inhibition of proinflammatory cytokines and/or NF-κB pathways. In the present study, it was demonstrated that oral treatment with hypoxoside ameliorated the colitis induced by *B. hyodysenteriae.* The anti-inflammatory effect of hypoxoside correlated with the downregulation of genes regulated by the NF-κB pathway in the colonic mucosa rather than displaying a direct anti-microbial effect.

MAMTERIAL AND METHODS

Animals. Eight to nine week old, C3H/HeOuJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All animal-related procedures were approved by the Animal Care and Use Committee at Iowa State University.

Bacteria. *Brachyspira hyodysenteriae* strain B204 was grown anaerobically at 37 ^{o}C in trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD) supplemented with 5 % horse serum (HyClone Laboratories, Logan, UT), 0.5 % yeast extract (BBL), 0.05 % L-cysteine (Sigma, St. Louis, MO) and 1 % VPI salt solutions. Highly motile, log phase cultures were used for all challenge inoculation. The concentration of bacteria was determined using a Petroff-Hausser counting chamber (Hausser Scientific, Horsham, PA).

Forty-eight hours prior to bacterial challenge, the diet was switched from conventional rodent chow (CRC) to Harlan Teklad 85420 (TD) diet (Harlan Sprague Dawley, Madison, WI) which had been previously shown to induce more consistent lesions following *B. hyodysenteriae* infection than CRC did (18). The TD diet alone had no deleterious effects on the mice or upon the intestinal mucosa (data not shown). Mice were maintained on the TD diet for the remainder of the experiment. Four hours prior to bacterial challenge, mice were fasted. Mice were orally inoculated with 1 x 10^{8} *B. hyodysenteriae* in 0.5 ml culture broth. The TD diet was returned to the cages immediately after inoculation. Control mice were inoculated with 0.5 ml culture broth without bacteria. *B. hyodysenteriae* colonization was confirmed bacteriologically at the termination of the experimental period.

Hypoxoside. Hypoxoside was a gift from Dr. Anthony Allison (Dawa Corp., Belmont, California). Fresh hypoxoside solution was made by dissolving hypoxoside in sterile

deionized water to a concentration of 60 mg/ml. Using a 22 gauge feeding needle, each mouse was orally intubated with 0.25 ml of the hypoxoside solution (15 mg) or water each day beginning seven days prior to challenge.

Experimental design. Mice were randomly assigned into four experimental groups. The first group was designated as "Control", which did not receive either *B. hyodysenteriae* challenge or hypoxoside treatment; the second group was designated as "Hypox only", which only received the hypoxoside treatment but not the *B. hyodysenteriae* challenge; the third group was designated as "Hypox + B. hyo", which received both the hypoxoside treatment and the *B. hyodysenteriae* challenge; the fourth group was designated as "B. hyo only", which received the *B. hyodysenteriae* challenge but not the hypoxoside treatment.

Mice in "Hypox only" and "Hypox + B. hyo" groups were treated with hypoxoside beginning seven days prior to *B. hyodysenteriae* challenge and the treatments continued daily for another seven days postinfection (DPI). Mice in "Hypox + B.hyo" and "B. hyo only" were challenged with *B. hyodysenteriae* on experimental day seven. The experiment was terminated seven DPI.

Gross and histological inflammatory lesion score. Gross and histological inflammatory lesion scores of ceca were evaluated as previously described (20). Briefly, gross inflammatory lesions were scored 0 to 3 as follows: cecal atrophy and excess intraluminal mucus = 3; atrophy and excess intraluminal mucus localized to the cecal apex = 2; increased cecal mucus with no evidence of atrophy = 1; and no gross lesions = 0. For histological lesions, cecal tissues were formalin-fixed, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E). Stained tissue sections were scored by a pathologist (Dr. J. M. Hostetter, Department of Veterinary Pathology, Iowa State University) in a blinded fashion. Histological scores were based on the severity of mucosal epithelial damage, degree of lamina propria cellular infiltrate, and architectural distortion.

Myeloperoxidase (MPO) activity assay. The assay measuring MPO was modified from a previously published method (39). Briefly, ceca were collected, homogenized, and sonicated in phosphate buffered saline (PBS) containing 0.1 mM phenylmethanesulphonylfluoride (PMSF). The resulting lysate was centrifuged and supernatant was aliquoted into a microtiter plate. MPO activity was determined

colorimetrically using 3,3', 5,5'-tetramethylbenzidine(TMB) as a substrate at an optical density of 405 nm. Serial dilutions of freshly prepared neutrophils recovered from murine peripheral blood were used to generate standard curves.

Pathway-specific microarray analysis of NF-κB -associated genes. At necropsy, murine cecal samples were collected and stored in RNAlater (Ambion, Austin, TX). Total RNA was isolated using the RNeasy Mini Kit and RNase-Free DNase according to the manufacture's protocol (Qiagen, Valencia, CA).

Oligo GEArray® Mouse NF-κB Signaling Pathway Microarray (Superarray, Frederick, MD) were used to compare transcript levels of NF-κB-associated genes in different experimental groups. Three milligrams of total RNA was converted into cRNA, hybridized to each array and detected by chemiluminescence according to the manufacturer's protocols. The array signals were captured by CCD camera and analyzed using "GEArray Expression Analysis Suite" (Superarray). Expression of each gene was normalized to housekeeping genes. The genes affected by hypoxoside treatment were selected by comparing the gene expression levels of "B. hyo only" group to "Hypox + B. hyo" group. The genes displaying a two or more fold change between these two groups were analyzed.

Active NF-κB p65 quantification. Activation of NF-κB within the ceca mucosa was measured using the TransAM NF-κB p65 Chemi Transcription Factor Assay Kit (Active Motif, Carlsbad, CA). Cecal tissues were homogenized in ice-cold Complete Lysis Buffer, incubated on ice for 30 minutes and then centrifuged at 10,000 x *g* for 10 minutes. The supernatants were collected and total protein concentrations were measured by Bradford colorimetric assay. To evaluate NF-κB activity, each protein extract (50 μg) was incubated in separate wells of a 96-well plate that contained immobilized nucleotide sequences for the consensus NF-κB binding site (5′-GGGACTTTCC-3′). Detection of NF-κB binding activity in each tissue extract was detected by chemiluminescence according to the manufacturer's directions.

Measurement of intestinal epithelial cell proliferation. After administration *in vivo*, 5-bromo-2-deoxyuridine (BrdU) can be incorporated into replicating cells (S phase of cell cycle) and this approach was used to assess mucosal epithelial cell proliferation in the cecal mucosa of mice in each of the four experimental groups. One hour before necropsy, each

mouse received an intraperitoneal injection of 0.5 ml PBS containing 2.5 mg BrdU (Boehringer Mannheim Corporation, Indianapolis, IN). At necropsy, ceca tissues were collected, fixed in formalin, and then embedded in paraffin. Paraffin-embedded tissue were routinely processed and sectioned for the purpose of immunohistochemical staining using the ZYMED ® BrdU Staining Kit (Invitrogen, Carlsbad, CA). Proliferation of mucosal epithelial cells in ceca was assessed microscopically by calculating the percentage of BrdU-stained epithelial cells in the cecal epithelium. Only those crypt units visible in their entire length (i.e., luminal shoulder to glandular base) were analyzed and a minimum of 5 crypts were counted.

Quantification of the number of *Brachyspira hyodysenteriae* in the ceca of infected mice. DNA was extracted from 100 mg cecal contents using QIAamp DNA stool Mini Kit (Qiagen). Quantitative PCR protocols to measure the number of *B. hyodysenteriae* were developed and optimized to detect the gene nicotinamide adenine dinucleotide hydride (NADH) oxidase (*nox*) of *B. hyodysenteriae* (26). The respective primer pairs were: H1 (forward primer) (5'-ACTAAAGATCCTGATGTATTTG-3') and H2 (reverse primer) (5'-CTAATAAACGTCTGCTGC-3'). DNA was amplified using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen) under the following conditions: 95 ℃ for 10 minutes, followed by 35 cycles at 95 ℃ for 30 seconds, 58 ℃ for 90 seconds, 68 ℃ for 2 minutes, followed by a final cycle at 68 ℃ for 10 minutes in Rotor-Gene 3000(Corbett Research, Mortlake**,** Australia). Standard curves of DNA encoding NADH oxidase were made by using DNA from a series of *B. hyodysenteriae* culture dilutions. The correct sizes of the amplified PCR products were confirmed by gel electrophoresis. The specificity of this primer pair was also confirmed by the absence of PCR products amplified from the DNA of ceca contents in mice without *B. hyodysenteriae* infection.

Statistical analysis. The results are expressed as mean ± standard error. The gross and histological lesions were analyzed using the robust Kruskal–Wallis non-parametric test while other data were analyzed by analysis of variance (ANOVA).

RESULTS

Hypoxoside ameliorated cecal inflammation lesions induced by *Brachyspira hyodysenteriae*. Macroscopically, *B. hyodysenteriae* infection of C3H mice induced cecal lesions characterized by cecal atrophy and increased intraluminal mucus. Compared to mice infected with *B. hyodysenteriae*, the challenged mice in the hypoxoside treated group (Hypox + B. hyo) had significantly ($P < 0.05$) lower cecal lesion scores indicating that hypoxoside treatment ameliorated or prevented the development of cecal lesions (Fig. 1 A, B). In addition, hypoxoside treatment significantly ($P <0.05$) prevented the weight loss induced by *B. hyodysenteriae* infection (Fig. 1C). Microscopically, hypoxoside treatment attenuated the characteristic mucosal hyperplasia, submucosal edema, erosion of epithelial cells, and leukocyte infiltration induced by *B. hyodysenteriae* infection (Fig. 2A, 2B).

Similarly, the inhibitory effects of hypoxoside treatment on inflammatory lesions were observed at several time points after *B. hyodysenteriae* infection (e.g., 3, 10, and 21 DPI) (data not shown). Collectively, these results showed that hypoxoside treatment can ameliorate the cecal inflammation induced by *B. hyodysenteriae* infection.

Hypoxoside treatment reduced the myeloperoxidase activity. The infiltration of leukocytes, especially neutrophils, is a common characteristic of acute inflammation. Myeloperoxidase is an enzyme found in the granules of neutrophils and has been commonly used as an indirect marker of neutrophil infiltration (22). Our experimental results showed that hypoxoside significantly attenuated the overproduction of MPO in the cecal tissue following *B. hyodysenteriae* infection (Fig. 3), indicating that the anti-inflammatory effects of hypoxoside also inhibited neutrophil infiltration. There was no significant difference in MPO levels among Control, Hypox only and Hypox + B. hyo groups, although the tissue samples from mice in the Hypox + B. hyo group tended to have lower MPO levels than those from the control group (P=0.08, Fig. 3)

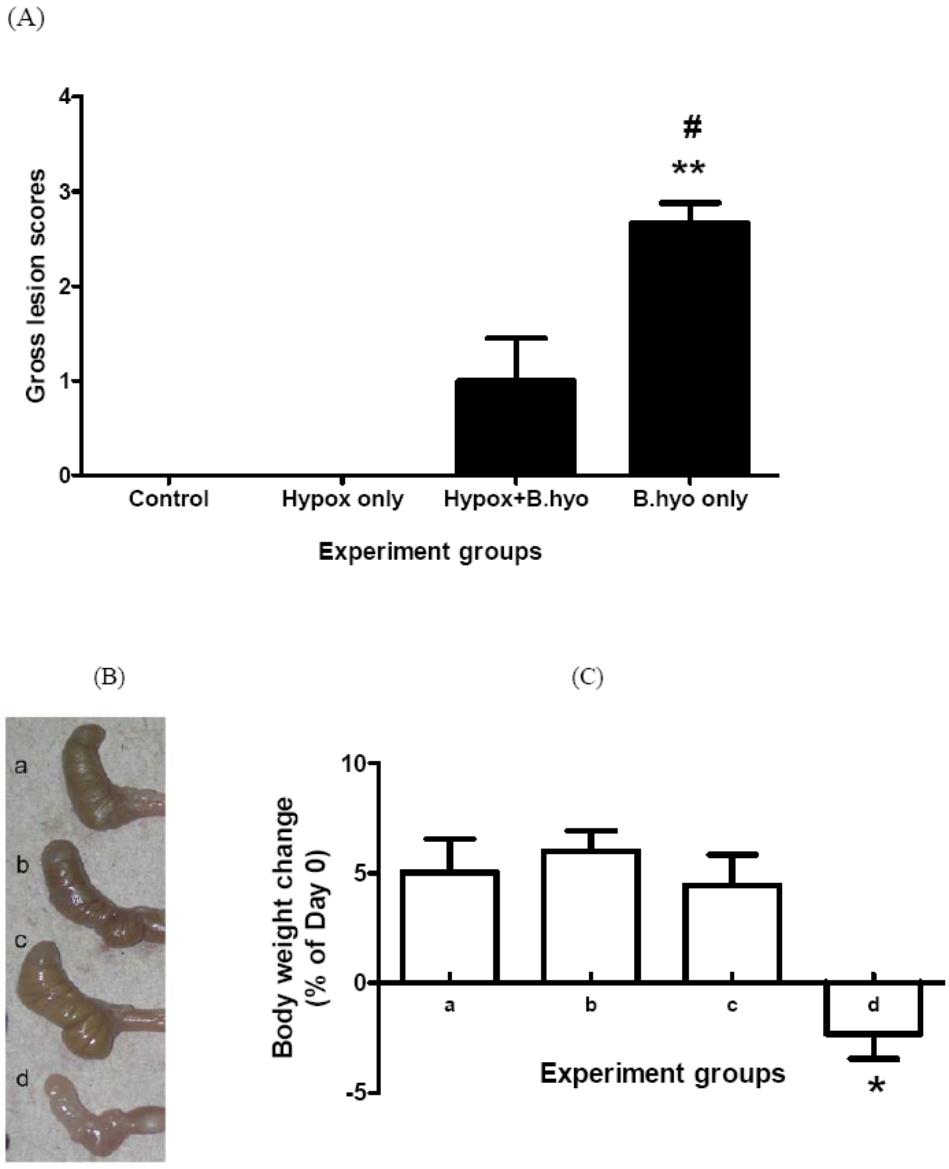

Figure 1. The effects of hypoxoside treatment on gross lesion and weight change induced by *Brachyspira hyodysenteriae* infection in mice. (A) Gross cecal lesion scores. ** $P < 0.01$ vs. Control, Hypox only; # $P < 0.05$ vs. Hypox + B. hyo. (B) Photomicrographs of representative murine ceca from each of the four treatment groups. (C) Changes in body weight on day seven after *B. hyodysenteriae* infection.*$P<0.05$ vs. all other groups. a: Control, b: Hypox only, c: Hypox + B. hyo, d: B. hyo only. n=5-6 mice. Hypox: Hypoxoside; B. hyo: *Brachyspira hyodysenteriae*.

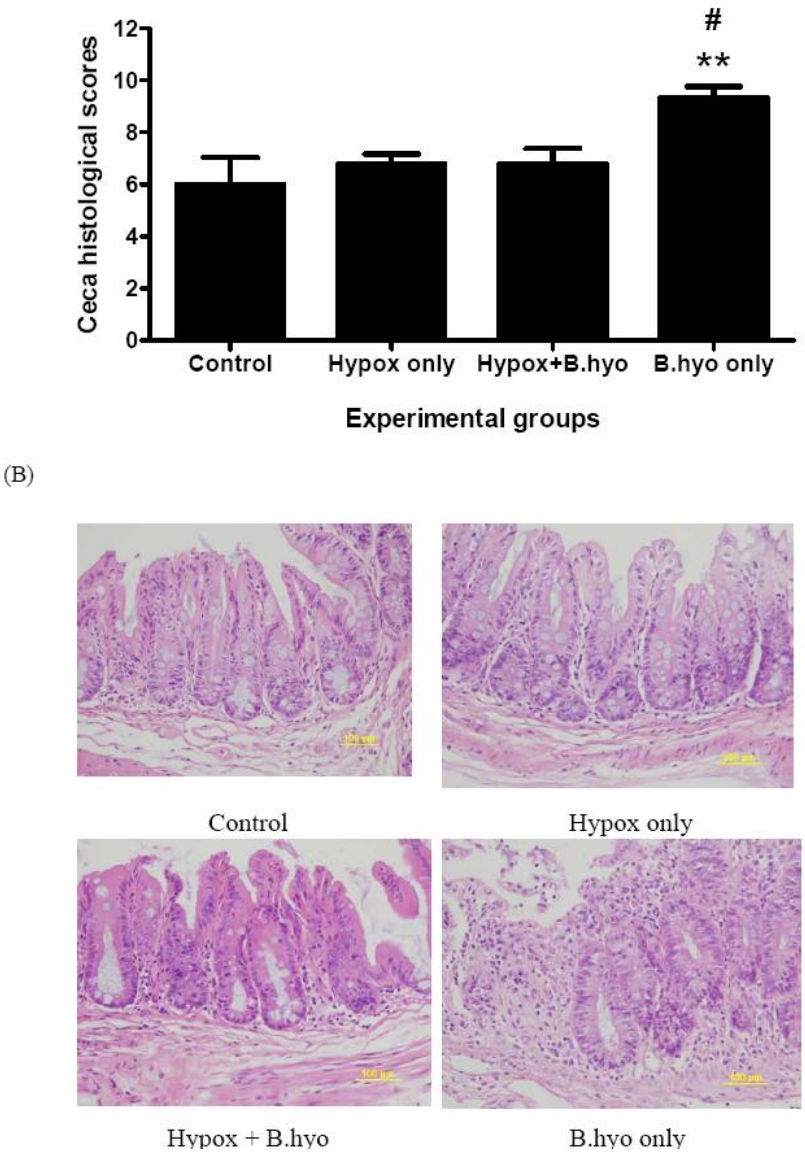

Figure 2. The effects of hypoxoside treatment on the cecal histological lesions induced by *Brachyspira hyodysenteriae* infection in mice. (A) Cecal histological lesion scores. ** $P < 0.01$ vs. Hypox only, Hypox + B. hyo; # $P < 0.05$ vs. Control. (B) Representative hematoxylin and eosin stained sections of the proximal cecum from mice in each treatment group. n=5 to 6 mice per group. Hypox: Hypoxoside; B. hyo: *Brachyspira hyodysenteriae*.

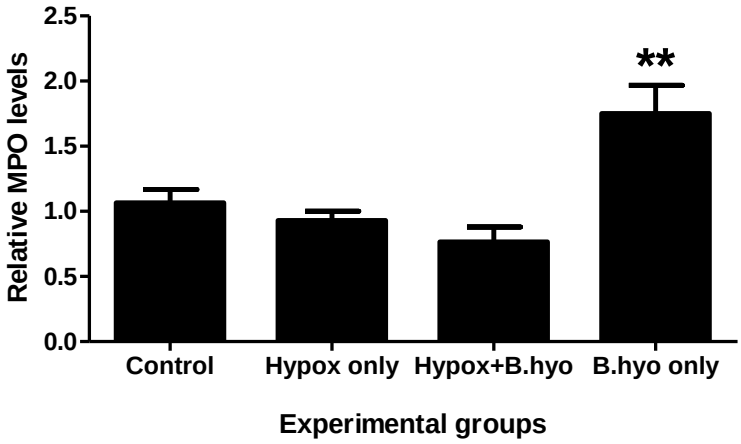

Figure 3. The effects of hypoxoside treatment on cecal myeloperoxidase (MPO) activity in mice. Ceca were homogenized, sonicated and supernatants clarified by centrifugation. The supernatant was aliquoted into a microtiter plates and MPO levels were determined colorimetrically at an optical density of 405 nm. ** $P < 0.01$ vs. all other groups. P=0.08 Control vs. Hypox + B. hyo. n=5-6 mice. Hypox: hypoxoside; B. hyo: *Brachyspira hyodysenteriae*.

NF-κB Pathway specific microarray identified the genes affected by hypoxoside treatment. The NF-κB pathway is multi-component signaling pathway regulating many aspects of cellular activities such as stress, inflammation, cell proliferation, and survival. The classical pathway of NF-κB activation induced by proinflammatory signals proceeds through IκB kinase (IKK)-mediated phosphorylation of cytoplasmic NF-κB inhibitors, the IκBs, leading to the formation and translocation of p50/p65 heterodimer into the nucleus (6, 34, 40). Recently, an alternative activation pathway for NF-κB, activated by stimulation of CD40, lymphotoxin-β receptors (Ltbr) or B-cell-activating factor and based upon the NF-κB inducing kinase (Nik)/IKKα-dependent processing of p100 to p52 was described (5, 31, 44, 49).This pathway preferentially leads to the formation and translocation of p52/RelB heterodimers into the nucleus.

To investigate the effects of hypoxoside on inflammation induced by *B. hyodysenteriae* infection, NF-κB pathway specific microarrays were employed to study the mucosal gene expression profiles after hypoxoside treatment. The results showed that *B. hyodysenteriae*

infection substantially increased the gene expression levels of a series of genes associated with the NF-κB pathway (Fig. 4 and Table 1). However, hypoxoside treatment reduced the expression levels of many genes upregulated following *B. hyodysenteriae* infection. Thus, the gene expression profile of mice in the Hypox + B. hyo group was similar to that of control mice (Fig. 4). Notably, the Hypox only group had the lowest level of gene expression among all groups tested, indicating that the dose of hypoxoside used reduced the homeostatic level of NF-κB signaling (Fig. 4). Additionally, the histological evaluation (Fig. 2) of the mice in the Hypox only group indicated that there were no deleterious effects of this treatment. By comparing the gene expression profile from B. hyo only and Hypox + B. hyo experimental groups, 20 genes were identified to be downregulated by at least 2 fold with the treatment of hypoxoside (Table 1).

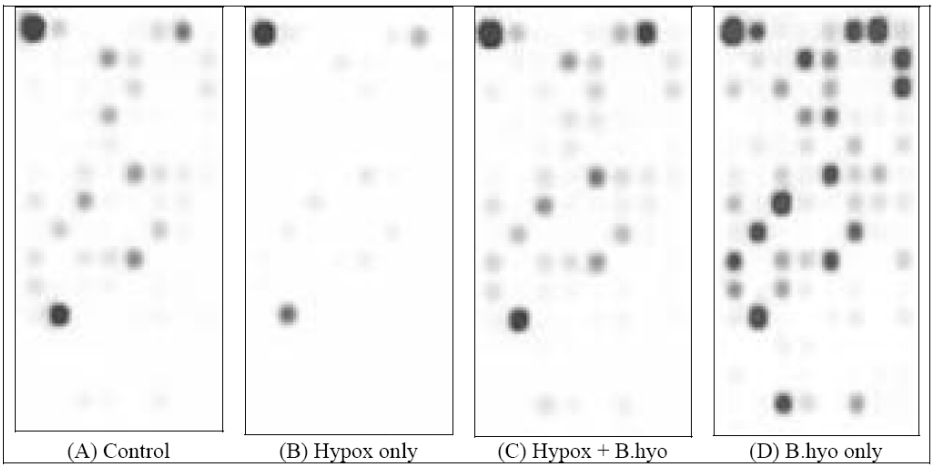

Figure 4. The effects of hypoxoside treatment on gene expression profiles regulated by the NF-κB pathway. Treatment with hypoxoside began seven days prior to challenge with *Brachyspira hyodysenteriae.* Cecal tissue was recovered from mice seven days after challenge with *B. hyodysenteriae.* Cecal RNA was converted into biotin-labeled cRNA and hybridized into NF-κB oligo nucleotide microarrays. Hybridization of gene-specific cRNA samples to their specific targets was detected by chemiluminescence as described in Materials and Methods. Hypox: Hypoxoside; B. hyo: *Brachyspira hyodysenteriae*.

Hypoxoside treatment downregulated a series of genes associated with the NF-κB signaling pathway. These included genes encoding cell surface receptors (e.g., *Ltbr*), adaptor proteins (e.g., *Myd88, Irak1*), IKB kinases (e.g., *Chuk (Ikka), Ikbke*), and transcriptional factors (e.g., *Nfkb1(p105) and Nfkb2(p100)).* In addition, the downregulated genes also included *Bcl3*, *Map3k14 (Nik)* and *Map3k7ip1 (Tab1)* all of which are upstream regulators of NF-kB activation. The precursor protein for p50, encoded by *p105*, forms a dimer with p65 in classical NF-κB pathway.The precursor protein for p52, encoded by *p100*, forms a dimer with RelB in alternative NF-κB pathway (40). *Bcl3* is co-activator of both p50 and p52 (4, 12). Notably, in addition to genes involved in classical NF-κB pathways (e.g., *Myd88, Ikka and p105*), the list of downregulated genes included genes involved in the alternative pathway (e.g., *Ltbr, Nik, Ikka and p100*) (40). These results indicate that hypoxoside treatment could downregulate expression of genes associated with different stages of both classical and alternative NF-κB signaling pathway. In addition, hypoxoside treatment also downregulated genes involved in MAPK signaling (e.g., *Mapk14 (P38), Fos*) and cell growth (e.g., *Akt1, Egr1*), indicating that the inhibition of MAPK signaling and inflammation-associated cell proliferation by hypoxoside treatment.

Quantification of the effect of hypoxoside on the levels of active NF-κB p65. Upon activation, transcriptional factor p65 can be dissociated from I-κ*B* and translocated into nucleus. To directly address the inhibition of NF-κB by hypoxoside treatment at the level of activated protein, the quantity of active NF-κB p65 protein was measured in extracts of cecal tissue. The results showed that *B. hyodysenteriae* infection significantly increased ($P < 0.05$) the amount of active NF-κB p65 protein in comparison to control mice (Fig. 5). Although the dual treated group (i.e., Hypox + B. hyo) had a higher level of p65 than that of the control group, they had significantly lower levels of active p65 than the tissue samples from the mice in the B. hyo only group ($P<0.05$), suggesting that hypoxoside treatment, at least partially, inhibited the activation of NF-κB p65.

Table 1. Hypoxoside treatment downregulated gene expression levels in the NF-κB pathway induced by *Brachyspira hyodysenteriae* infection.

Gene name[a]	Gene Bank ID	Fold change[b]
NF-κB /MAPK signaling		
Ltbr: Lymphotoxin B receptor	NM_010736	-3.65
Myd88: Myeloid differentiation primary response gene 88	NM_010851	-5.11
Irak1: Interleukin-1 receptor-associated kinase 1	NM_008363	-2.20
Chuk: Conserved helix-loop-helix ubiquitous kinase	NM_007700	-13.04
Ikbke:Inhibitor of kappaB kinase epsilon	NM_019777	-3.59
Bcl3: B-cell leukemia/lymphoma 3	NM_033601	-3.43
Nfkb1: Nuclear factor of kappa light chain gene enhancer in B-cells 1, p105	NM_008689	-4.91
Nfkb2: Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100	NM_019408	-2.44
Map3k14: Mitogen-activated protein kinase kinase kinase 14	NM_016896	-15.04
Map3k7ip1: Mitogen-activated protein kinase kinase kinase 7 interacting protein 1	NM_025609	-2.85
Mapk14: Mitogen activated protein kinase 14	NM_011951	-2.33
Fos: FBJ osteosarcoma oncogene	NM_010234	-6.01
Others		
Akt1: Thymoma viral proto-oncogene 1	NM_009652	-3.23
Casp8: Caspase 8	NM_009812	-3.55
Ecm1: Extracellular matrix protein 1	NM_007899	-2.21
Egr1:Early growth response 1	NM_007913	-8.69
Gja: Gap junction membrane channel protein alpha 1	NM_010288	-10.44
Litaf: LPS-induced TNF factor	NM_019980	-6.23
Ppm1a: Protein phosphatase 1A, magnesium dependent, alpha isoform	NM_008910	-4.25
Tollip: Toll interacting protein	NM_023764	-9.53

a. Gene expression levels of "Hypox + B. hyo" group were compared to those of "B. hyo only" group.

b. Fold change were defined as comparative gene expression levels between "B. hyo only" group and "Hypox + B. hyo" group. Negative fold changes represent the magnitude of gene expression hypoxoside treatment downregulated.

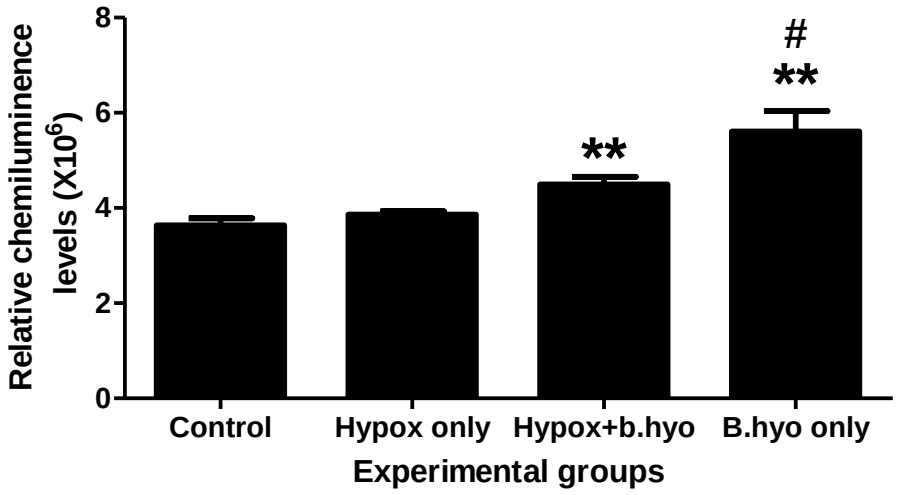

Figure 5. The effects of hypoxoside treatment on active cecal mucosal p65 protein levels in mice. Seven days postinfection, cecal tissues were collected and lysates were prepared as described in Materials and Methods. The supernatant were collected and incubated in a 96-well plate that contained immobilized nucleotide sequences for the consensus NF-κB binding site. Active p65 levels in cell extract of mouse ceca were measured by ELISA with chemiluminescent readout. * $P < 0.05$ vs. Control, # $P < 0.05$ vs. all other groups. n=5-8 mice. Hypox: Hypoxoside; B. hyo: *Brachyspira hyodysenteriae*.

Hypoxoside treatment reduced the intestinal epithelial cell proliferation. One of the key histological features of cecal lesions induced by *B. hyodysenteriae* infection is mucosal hyperplasia (18, 41)**.** To correlate the reduction of histological lesions and mucosal hyperplasia (Fig. 2) with regulation of cell growth/proliferation**,** epithelial cell proliferation was assessed by measuring BrdU incorporation (Fig. 6A, B). Immunohistochemical staining of tissue sections from control and hypoxoside treated mice revealed that there was about 10% proliferating epithelial cells at the base of crypts (Fig. 6A). The cecal tissue recovered from *B. hyodysenteriae* infected mice displayed about approximately a three-fold increase in the number (Fig 6A) of proliferating cells that extended above the base of the crypts. More importantly, hypoxoside treatment significantly ($P<0.01$) reduced the number of proliferating cells within the cecal epithelium following infection with *B. hyodysenteriae* to a level similar to that observed for control mice and mice treated with hypoxoside alone (Fig. 6A).

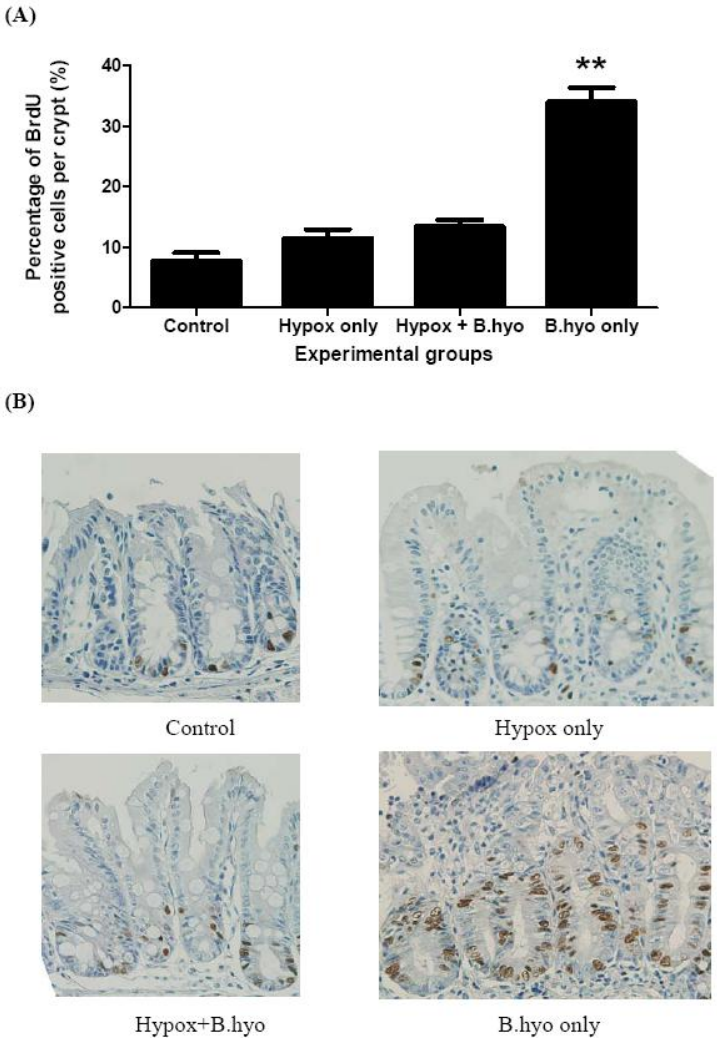

Figure 6. The effect of hypoxoside treatment on cecal epithelial cell proliferation in mice shown by BrdU staining. Seven days postinfection with *Brachyspira hyodysenteriae*, mice received an intraperitoneal injection of BrdU one hour before necropsy. As described in Materials and Methods, cecal tissues were processed and sectioned for immunohistochemical staining to detect BrdU incorporation. Data is presented as the percentage of proliferating mucosal epithelial cells (A) The quantitative analysis of percentage of BrdU positive cells in crypts. For each group, five complete crypt units per mouse were included in the data anlysis ** $P<0.01$ vs. all other groups. (B) Photomicrographs of representative murine cecal BrdU staining. Magnifications of pictures are 40X. n = 5 to 6 mice per group. Hypox: Hypoxoside; B. hyo: *Brachyspira hyodysenteriae*.

Evaluation of the antibacterial effects of hypoxoside on *B. hyodysenteriae*. To determine whether the anti-inflammatory effect of hypoxoside is associated with potential anti-microbial activity, the ability of hypoxoside to inhibit *in vitro* bacterial growth was evaluated for multiple bacterial species. Addition of hypoxoside (0 to 1 mg/ml) to *in vitro* culture medium did not inhibit the growth of *Lactobacillus murinus, L. acidophilus, Bacteroides distasonis, Escherichia coli, Enterococcus faecalis, Staphylococcus epidermidis,* or *Helicobacter bilis* (data not shown). With respect to *in vitro* inhibition of *B. hyodysenteriae* growth, there was an extension of the lag phase of growth in broth cultures, but the treated cultures eventually grew as well as the untreated cultures (data not shown). In addition, the number of *B. hyodysenteriae* in the cecal contents of *B. hyodysenteriae* mice (with or without hypoxoside treatment) was quantified using quantitative PCR. The result showed that hypoxoside treatment did not significantly ($P > 0.05$) alter the number of *B. hyodysenteriae* in the ceca of infected mice (Fig.7). These data suggested that the effect of the hypoxoside treatment (i.e., reduction of mucosal inflammation) was not the result of anti-microbial activity on the resident flora or on *B. hyodysenteriae*, but on the host inflammatory response.

DISCUSSION

Hypoxoside, a traditional medicine of southern Africa, has been used as a drug for a series of human aliments for centuries (38). One of medicinal properties associated with the use of hypoxoside has been its anti-inflammatory activity. Although *in vitro* studies showed that rooperol, the active component of hypoxoside, blocks the production of inflammatory mediators (3, 14, 15, 25), no *in vivo* experiments have been performed to demonstrate the anti-inflammatory property of hypoxoside on colitis. In this paper, it was shown that oral treatment of mice with hypoxoside could significantly reduce the colitis induced by *B. hyodysenteriae*. In addition, the inhibitory effects of hypoxoside on the bacterial-induced colitis correlated with the downregulation of the NF-κB pathway in the colonic mucosa rather than exerting a direct anti-microbial effect.

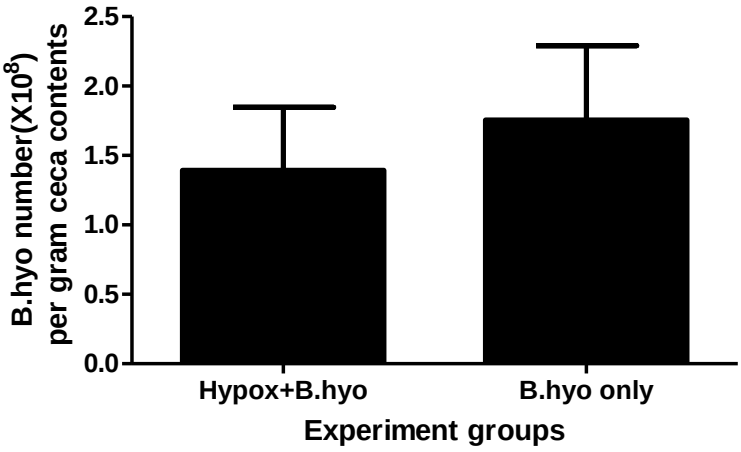

Figure 7. The effects of hypoxoside treatment on the number of cecal *Brachyspira hyodysenteriae* in mice. At the termination of the experiment, cecal contents were collected from each mouse and used to extract DNA for bacterial quantification as described in Materials and Methods. DNA was extracted from separate cecal contents of mice in Hypox + B. hyo group and B. hyo only group. The bacterial numbers were quantified using real-time PCR with specific *B. hyodysenteriae* primers. Standard curves were generated by using DNA from pure culture *B. hyodysenteriae*. Bacterial numbers were then normalized based on a per gram equivalence. n = 6 mice per group. Hypox: Hypoxoside; B. hyo: *Brachyspira hyodysenteriae*.

Neutrophil infiltration is a hallmark of inflammatory reactions including colitis. It is one of several host defense mechanisms employed to counteract the deleterious effects of bacterial infections. Inflammatory mediators released by neutrophil include nitric oxide, reactive oxygen species, and prostaglandins which can contribute to tissue damage (e.g., submucosal edema and epithelial erosion) (27). The amelioration of tissue lesions by treatment with hypoxoside correlated with the reduction of neutrophil infiltration as assessed microscopically and by reduction in myeloperoxidase activity (Fig. 3). The inhibition of neutrophil activity are consistent with previous studies that showed that blockage of CD18 or direct depletion of neutrophils attenuated/abrogated lesion severity in mice infected with *B. hyodysenteriae* (41).

Transcription factor NF-κB is a key regulator of the inducible expression of many genes associated with intestinal inflammation, including pro-inflammatory cytokines/chemokines, growth factors, adhesion molecules, cell surface receptors, transcription factors and others (35). Excess or inappropriate activation of NF-κB has been observed in IBD patients and in various animal models of IBD; in addition, drugs inhibiting NF-κB pathway have been used to treat IBD (2, 9). Consistent with the role of NF-κB activation in IBD, the present studies suggest that hypoxoside treatment directly inhibited the expression of genes associated with NF-κB signaling cascades. Meanwhile, the levels of activated p65 decreased as a consequence of hypoxoside treatment. These results indicate that inhibition of NF-κB signaling might contribute to the anti-inflammatory effects of hypoxoside. In addition, NF-κB participates in the regulation of cell proliferation and death in many cell types and typically promotes the cell proliferation and suppresses apoptosis (28). Consistent with this, genes associated with cell proliferation were found to be downregulated (Table 1) and epithelial cell proliferation based on BrdU incorporation (Fig. 6) was attenuated after hypoxoside treatment.

NF-κB signaling includes two distinct pathways (i.e., classical and alternative signaling pathways). In contrast to classical pathway, the alternative NF-κB pathway is generally involved in biological processes including secondary lymphoid organ development, initiation of adaptive immunity, cell survival, and proliferation (40). Surprisingly, it was observed that, in addition to classical NF-κB signaling, hypoxoside had the capability to attenuate the activation of alternative pathways following *B. hyodysenteriae* infection. Differential contribution of the two NF-κB activation pathways to IBD pathogenesis is still unknown. However, activation of alternative NF-κB signaling was shown in animal models of ulcerative colitis (17).

Notably, the amount of active p65 detected in the mucosa of mice that had been treated with hypoxoside and challenged with *B. hyodysenteriae* was significantly ($P<0.05$) higher than that detected for the control group (Fig. 5). This indicated that; 1) inhibition of NF-κB activity alone may not be solely responsible for the observed anti-inflammatory effects of hypoxoside, or 2) the dose of hypoxoside used was not sufficient to completely inhibit the activation of NF-κB. In this regard, rooperol has been shown to reduce the binding activity of

transcriptional factors such as activating protein 1(AP-1) *in vitro*, indicating that AP-1 might also play a role in hypoxoside effects (15). Consistent with these observations, it was shown that hypoxoside downregulated the *Fos* gene which encodes an essential component of AP-1 (Table 1).

Unexpectantly, there was no significant downregulation of the proinflammatory cytokines such as IL-1β, TNF-α detected after hypoxoside treatment (data not shown). The inability to detect changes in the expression of mRNA specific of the pro-inflammatory cytokines may be related to the experimental end point chosen for these studies (i.e., 7 DPI). Preliminary data relative to these studies indicated that the expression of proinflammatory cytokine genes, such as TNF α, peaked between two and three DPI and rapidly returned to background levels (data not shown).

The *in vitro* evaluation of bacterial growth in the presence of hypoxoside and *in vivo* quantiation of *B. hyodysenteriae* in cecal contents supported the conclusion that the anti-inflammatory effects of hypoxoside were not dependent upon a bactericidal activity. The lack of anti-microbial effects by hypoxoside is in contrast to other studies, which reported that hypoxoside inhibited the growth of *Escherichia coli* and *Staphylococcus aureus,* albeit at levels of 4 mg/mL (25, 47). The reasons for these differences in the assessment of the anti-bacterial activity of hypoxoside are currently unknown and may be associated with different bacterial culture conditions and doses of hypoxoside used in the studies (e.g., 1 mg/mL v.s. 4 mg/mL).

Hypoxoside could be a new candidate for treatment of colitis. The toxicity of hypoxoside was shown to be low or absent (45) which is consistent with the long-time usage of this drug in southern Africa. In addition to anti-inflammatory effects, hypoxoside has also been perceived to provide nutritional and anti-oxidant benefits that might be helpful for maintaining overall health (32, 33). More importantly, hypoxoside appears to have exerted the anti-inflammatory effects locally (i.e., mucosa) and not systemically as demonstrated by the fact that no hypoxoside or rooperol was found in sera of individuals treated with oral hypoxoside (23). This would suggest that the therapeutic use of drugs such as hypoxoside would not have adverse systemic effects; this should be viewed as a promising outcome,

given the fact that long-term usage of many current anti-inflammatory drugs (e.g., corticosteroids) has systemic adverse effects (50).

In summary, this paper demonstrated for the first time that a plant extract, hypoxoside, could ameliorate bacterial-induced colitis and might provide therapeutic benefits for other forms of colitis. The anti-inflammatory effect of hypoxoside correlated with the downregulation of genes regulated by the NF-κB pathway within the colonic mucosa rather than displaying a direct anti-microbial effect.

ACKNOWLEDGEMENTS

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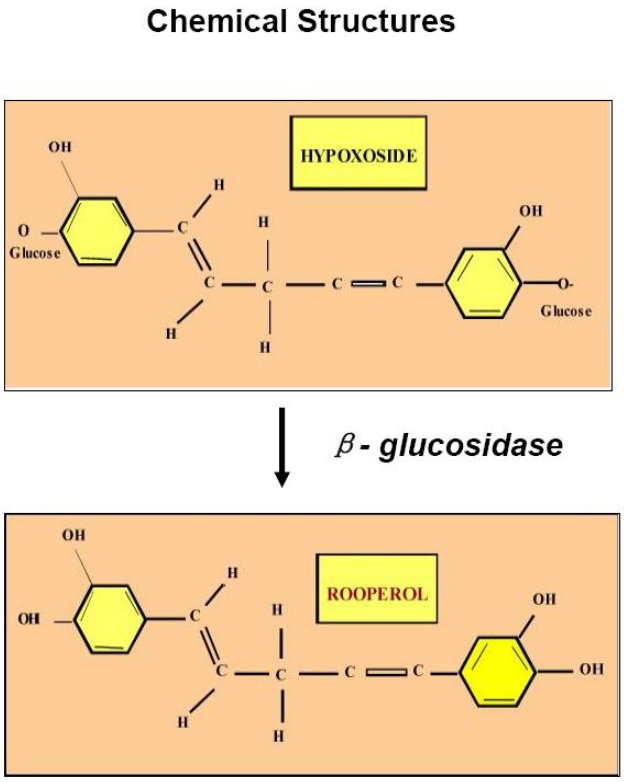
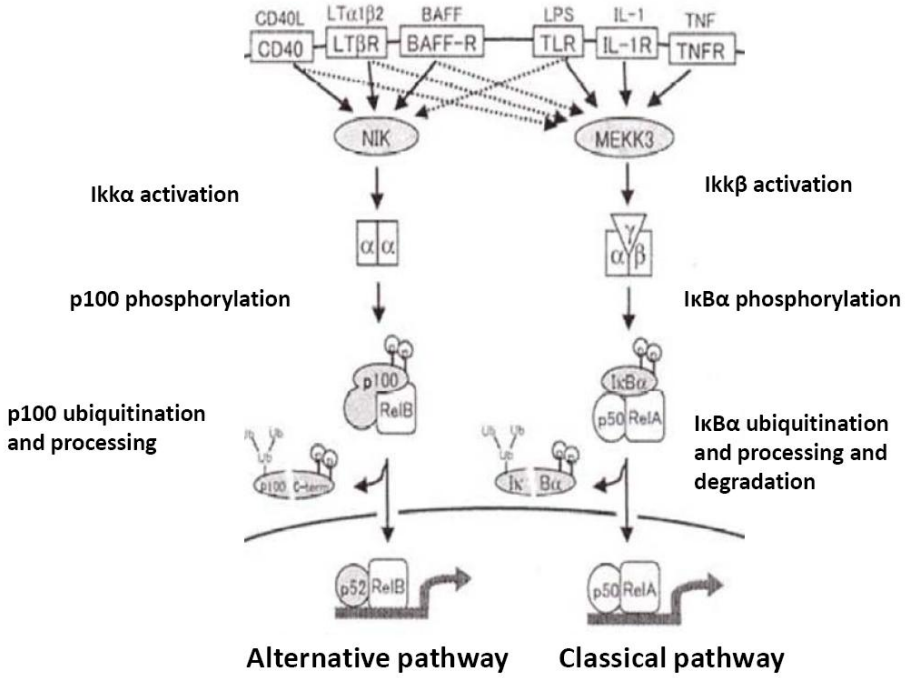


Supplemental Figure 1. The chemical structures of hypoxoside and rooperol. Hypoxoside can be converted into its active form rooperol by beta-glucosidase.

(**Drewes SE, Hall AJ, Learmonth RA, Upfold UJ**. Isolation of hypoxoside from *Hypoxis rooperi* and synthesis of [E]-1,5 Bis[3',4'-dimethoxypheny1] pent-1-en-1-ync. *Phytochemistry* 23:1313-1316, 1984.)

Gapd	Akt1	Atf1	Atf2	Bcl10	Bcl3	C3	Card10
Card11	Card14	Card4	Casp1	Casp8	Ccl2	Cflar	Chuk
Crebbp	Csf2	Csf3	Dusp1	Ecm1	Edaradd	Edg2	Egr1
Elk1	F2r	Fadd	Fos	Gja1	Hmgb1	Htr2b	Icam1
Ifna1	Ifng	Ikbkb	Ikbke	Ikbkg	Il10	Il1a	Il1b
Il1r1	Il6	Irak1	Irak2	Irf1	Jun	Litaf	Lta
Ltbr	Malt1	Map2k3	Map2k4	Map2k6	Map3k1	Map3k14	Map3k3
Map3k7	Map3k7ip1	Map3k7ip2	Map4k2	Mapk11	Mapk14	Mapk3	Mapk8
Myd88	Nalp12	Nfkb1	Nfkb2	Nfkbia	Pcaf	Plk2	Polr2a
Ppm1a	Ppp5c	Prkr	Raf1	Rel	Rela	Relb	Ripk1
Ripk2	Slc20a1	Smad3	Smad4	Stat1	Tbk1	Tgfbr1	Tgfbr2
Tlr1	Tlr2	Tlr3	Tlr4	Tlr6	Tlr7	Tlr8	Tlr9
Tnf	Tnfaip3	Tnfrsf10b	Tnfrsf1a	Tnfrsf1b	Tnfrsf5	Tnfrsf7	Tnfsf10
Tnfsf14	Tnfsf6	Tollip	Tradd	Traf2	Traf3	Traf5	Traf6
Vapa	Zap70	PUC18			AS1R2	AS1R1	AS1
Rps27a	B2m	Hspcb	Hspcb	Ppia	Ppia	BAS2C	BAS2C

Supplemental Figure 2. Gene table of NF-κB signaling pathway-specific microarray. (http://www.superarray.com)



Supplemental Figure 3. Illustration of classical and alternative NF-κB signaling pathway. In classical NF-κB activation, signaling is typically triggered through TNFR, IL-1R or TLR. Signals mediated by MAP/ERK Kinase kinase 3 (MEKK3) and IKKβ finally result in the degradation of I-κBα and translocation of the RelA/p50 homodimer to the nucleus. In the alternative NF-κB activation pathway, signals triggered via CD40, LTβR, or BAFF-R are mediated by NIK and IKKα, which leads to p100 processing and the translocation of p52 dimers into the nucleus.

(**Nishikori M**. Classical and alternative NF-κB activation pathways and their roles in lymphoid maligancies. *J. Clin. Exp. Hematopathol* 45(1):16-24, 2005.)

Summary of Bacterial Species Evaluated and Results

Bacteria Tested	Plate/Broth, Culture Conditions and Detection Method	Effect of Hypoxoside
Lactobacillus murinus (ASF 361)	broth, aerobic, OD	no observable effect
Lactobacillus acidophilus (ASF 360)	broth, aerobic, OD	no observable effect
Bacteroides distasonis (ASF 519)	broth, aerobic, OD	no observable effect
Clostridium cluster XIV (ASF 502)	plate, anaerobic, visual assessment	no observable effect
Low G+C, gram positive (ASF 500)	plate, anaerobic, visual assessment	no observable effect
Escherichia coli	broth, aerobic, OD & direct count	slight enhancement of growth
Enterococcus faecalis	broth & plate, aerobic, OD, direct count & visual assessment	no observable effect on growth, different hemolysis patterns on plates (see figure)
Enterobacter sp.	broth, aerobic, OD	no observable effect
Staphylococcus epidermitis	broth, aerobic, OD	no observable effect
Helicobacter bilis	plate, microaerophilic, visual assessment	no observable effect
Brachyspiria hyodysenteriae	broth & plate, anaerobic, direct count & visual assessment	slight decrease in cell numbers (24 hrs) in broth, decreased growth on plates (72 hrs)

Supplemental Table 1. The effects of hypoxoside on bacterial growth *in vitro*. Members of the Altered Schaedler Flora, *Brachyspira hyodysenteriae* and selected bacterial species isolated from healthy C3H/HeOuJ mice were evaluated for *in vitro* growth after inoculation into broth media containing differing doses of hypoxoside. Broth tubes or plates contained concentrations of hypoxoside: 0, 15.6, 62.5, 250, or 1000 mg/ml. Growth was monitored for 12 to 24 hours (broth cultures) up to 72 hours (plate cultures). Optical Density (OD) or absorbance measurements were made using Spectronic 20 Spectrophotomer, direct counts were made with the aid of a Petroff-Hausser counting chamber. The results were summarized using three independent experiments.

CHAPTER 5. GENERAL CONCLUSION

Summary of results

Inflammatory bowel disease (IBD), a chronic and relapsing intestinal inflammation, affects about 3.6 million individuals in the United States and Europe and is reported to have a significant impact upon the cost of health care.[1-3] However, the etiology of IBD is still unknown and likely involves complex interactions among genetic susceptability, gut bacteria flora and host immune system.[4-6]

The characterization of mucosal gene expression profiles in different IBD animal models will potentially provide molecular insights into the mechanisms that initiate, perpetuate or ameliorate IBD development. In this dissertation, alteration of mucosal gene expression profiles induced by different agents including a mild bacterial pathogen, *Helicobacter bilis* or an anti-inflammatory drug, hypoxoside) were characterized. The association between differential mucosal gene expression and modulation of colitis development was studied.

As a mild pathogen, *Helicobacter bilis* has been shown to induce and/or accelerate the development of colitis in immune-deficient rodents.[7-11] In an immunocompetent host, *H. bilis* colonization did not induce demonstrable inflammation yet triggered persistent antibody and cytokine responses to commensal bacteria.[12, 13] These results indicate that colonization of a novel bacterium such as *H. bilis* into gut might alter mucosal homeostasis and increase host susceptibility to colitis. However, the molecular and cellular mechanisms governing the effects of *Helicobacter bilis* colonization on the host mucosal homeostasis and colitis development remain unknown.

Using microarray analysis, changes in mucosal gene expression potentially contributing to an increased susceptibility to colitis were evaluated in gnotobiotic C3H mice at 15, 30 and 45 days following *H. bilis* colonization. Our results showed that *H. bilis* colonization induced marked upregulation of genes associated with protein metabolism, immune responses, and downregulation of genes associated with fatty acid metabolism and detoxification which peaked at 15 days postinfection. Interestingly, genes with similar biological functions follow similar temporal expression change patterns. For instance,

expression levels of genes associated with proteolysis (e.g., *Gzma* and *Mcpt1*) and innate immune responses (e.g., *Cd14*) went up at 15 DPI and returned back to control levels at 30 and 45 DPI. In contrast, expression levels of genes associated with glycoprotein synthesis (e.g., *Fut2* and *B3galt5*) and detoxification (e.g., *Cyp4b1*and *Ugt8a*) were upregulated or downregulated, respectively, at 15 DPI and stayed differentially expressed at 30 and 45 DPI, although the magnitude of gene expression over control mice decreased at these time points. Interestingly, some of these genes (e.g., *Fut2, B3galt5, Ceacam12, Cyp4b1, and Ugt8a*) were also differentially expressed in other colitis models including dextran sodium sulfate (DSS) and *Brachyspira hyodysenteriae* –induced colitis.

To directly determine if *H. bilis* colonization increased the sensitivity to DSS-induced colitis, mice were colonized with *H. bilis* for 6 weeks and then treated with a low dose of dextran sodium sulfate (DSS, 1.5 %) for five days followed by a four day restitution period. Our results demonstrated that *H. bilis* colonization increased the severity of colitis induced by DSS. Mucosal gene expression profiles showed that mice colonized with *H. bilis* plus treated with DSS had significant increases in the expression levels of genes associated with activation of immune cells (e.g., *Cd28, Tnfsf13b/Baff*) and recruitment of immune cell including the adhesive molecules(e.g., *Itgb2/Cd18, Itgam/Cd11b* and *ItagL/Cd11a*), chemokines/chemokine receptors(e.g., *Ccl8, Ccr5*and *Ccr10*). Consistently, increased infiltration and/or proliferation of T cells, B cells and macrophages in the intestine mucosa and/or MLN were observed in mice treated with *H. bilis* + DSS in comparison to mice treated with DSS alone. The upregulation of the cell activation markers (e.g., *Cd28, Tnfsf13b/BAFF*), pro-inflammatory cytokines *IL17*, and significant increased antibody responses to *H. bilis* and ASF suggest the T cell and B cell were activated. Surprisingly, neutrophil infiltration was not observed. In addition, the detoxification-associated genes *Cyp4b1* and *Ugt8a* were found to be downregulated in mice treated with *H. bilis* + DSS in comparison to mice treated with DSS alone.

Lastly, we used another other IBD model, *Brachyspira hyodysenteriae* - induced colitis. *Brachyspira hyodysenteriae* is the causative agent of swine dysentery and induces a characteristic mucosal inflammatory response resulting in pronounced typhlocolitis in mice and swine.[14, 15] Hypoxoside has been reported to be an anti-inflammatory drug extracted from

an African medicinal plant, *Hypoxis hemerocallidea.*[16] *In vitro* studies have shown that hypoxoside inhibits the secretion of pro-inflammatory cytokines and the nuclear binding activity of NF-κB.[17-20] However, no *in vivo* experiments have been performed to demonstrate the anti-inflammatory property of hypoxoside on colitis.

In this dissertation, our results showed that oral treatment of hypoxoside could ameliorate the colitis induced by *B. hyodysenteriae* infection, which was demonstrated by the reduced gross and histological lesions, reversed weight loss. The anti-inflammatory effects of hypoxoside were suggested to be associated with the reduction of neutrophil infiltration, downregulation of genes involved in NF-κB signaling pathways and decrease in mucosal active NF-κB subunit p65 protein levels and epithelial cell proliferation, but not anti-microbial activity.

Discussion and recommendation for future study

Downregulation of detoxification-associated genes

One of the key observations in this dissertation is that multiple detoxification-associated genes were identified to be downregulated following the colonization of gnotobiotic C3H mice with *Helicobacter bilis.*

Three general phases of detoxification processes can be defined. Phase 1 reactions include redox reactions via cytochrome P450 (CYP) enzymes, [21]hydration by carbonic anhydrases,[22] and hydrolysis of compounds by carboxylesterases.[23] Phase 2 reactions include the sulfation,[24] glucuronidation,[25] and glutathione[26] conjugation of reactive intermediates, leading to more hydrophilic molecules, which can be more easily exported from cells. A specific set of ABC transporter (i.e., multi-drug resistant transporter) proteins located at the basolateral or apical surface of intestinal epithelial cells mediates the efflux of unconjugated or conjugated compounds back into the gut lumen.[27]

Detoxification and biotransformation of luminal agents is an important protective function of intestinal epithelial cells.[28] Downregulation of detoxification genes has been described for several animal models of IBD (e.g., DSS-induced colitis, $TCR\alpha^{-/-}$, and transfer of CD45RBhi T cells) and human patients. [29, 30] Loss or dysregulation of detoxification and

biotransformation processes by the intestinal epithelium may contribute to the initiation and progression of IBD.[28-30]

Our microarray results showed that *H. bilis* colonization can induce significant downregulation of phase 1 enzymes including carboxylesterase *Ces1, Ces3,* cytochrome P450 gene family *Cyp4b1, Cyp4f1,* and epoxide hydrolase *Ephx2* and phase 2 enzymes including glucuronyl transferase*Ugt8* and glutathione transferase *Gstt1*. *Mdr1a* (multiple drug resistant 1a, or ATP-binding cassette, sub-family B, member 1A) was also found to be downregulated at 15 DPI after *H. bilis* colonization (data not shown). In addition, the detoxification transcriptional factor, Car (constitutive androstane receptor, also refered to Nr1i3) was downregulated after *H. bilis* colonization. Importantly, most of these detoxification genes stayed downregulated at 30 and 45 DPI in comparison to control. Collectively, *H. bilis* colonization resulted in systemic downregulation of a series of genes associated with detoxification processes. Notably, the downregulation of detoxification – associated genes only occured in the intestines of mice challenged with live *H. bilis,* but not dead *H. bilis*. Our preliminary data showed dead *H. bilis* challenge induced the antibody responses to *H. bilis* but did not alter the expression of detoxification genes *Cyp4b1* and *Ugt8a*, indicating that alteration of mucosal function, rather than antigen exposure alone, may be responsible for expression change of detoxification genes (data not shown).

We then asked if the downregulation of detoxification genes is specific for *H. bilis* colonization. We tested the other colitis models including DSS and *Brachyspira hyodysenteriae* –induced colitis based on 2% DSS for 5 days, 7 days after 10^8 *B. hyodysenteriae* infection in gnotobiotic C3H mice. The similar gene change patterns were identified for these two colitis models, based on the significant downregulation of genes *Cyp4b1* and *Ugt8a*.

One of the critical questions for downregulation of detoxification genes is whether it is one of the causes/ initiators of IBD or it is simply a response secondly to the inflammation. Pro-inflammatory cytokines such as TNFα have been shown to downregulate the expression of detoxification genes.[31] We found that hypoxoside, an anti-inflammation drug, can reverse the downregulation of detoxification gene *Cyp4b1,* which was correlated with the amelioration of inflammatory lesions induced by *Brachyspira hyodysenteriae* and inhibition

of NF-κB signaling pathway (data not shown). These results seem to support that the downregulation of detoxification genes is a result of inflammation or is secondary to inflammation. On the other hand, other evidence supports that dysregulation of the detoxification process may be a cause or initiator of IBD. Mice with deficiency in *Mdr1a*, one of critical genes in detoxification, spontaneously develop colitis [32]and *Mdr1a* polymorphism has been shown to be strongly associated with ulcerative colitis.[33, 34] Interestingly, our results showed that the fold change of downregulation of detoxification genes in acute inflammation induced by DSS or *B. hyodysenteriae* were actually lower than that of mild inflammation induced by *H. bilis* at 15 DPI (Chapter 2 Figure 5 and 6). This result indicates the downregulation of detoxification might not be directly correlated with the degree of inflammation.

It would be very important and interesting to directly test if dyregulation of detoxification genes is a cause or a result of intestinal inflammation. Specific deletion/knock-in of some of those important genes such as *Cyp4b1*, *Ugt8a* or some transcriptional factor such as nuclear receptor *Car* would provide a definite answer. It is also worthy to try agonist for relevant nuclear receptors and study if the activation of detoxification process would prevent the intestinal inflammation. Interestingly, one recent study showed that activation of another detoxification-associated nuclear factor *Pxr* (pregnane x receptor) could ameliorate the DSS-induced colitis, indicating dysregulation of detoxification might be the cause of IBD.[35] Given the fact that downregulation of *Pxr* has been identified in patients in ulcerative colitis[29] and *Pxr* polymorphism was shown to be strongly associated with IBD,[36] the agonist for detoxification transcriptional factor might be a potential therapeutic strategy to treat IBD patients.

Another fundamental question about the detoxification-associated genes is whether those genes are derived only from epithelial cell. Is it possible that other cells in lamina propria also express these genes, given the fact that the whole tissue mucosa was used in our microarray studies? Notably, one microarray study which used intestinal epithelial cells also identified downregulation of a series of detoxification-associated genes, indicating detoxification genes might be from epithelial cells.[30] The *Mdr1a* gene has been identified to be expressed in epithelial cells and lymphocytes, hematopoietic cells. However, defect of

Mdr1a in the intestinal epithelial cells is the principle cause of colitis.[37, 38] Ongoing studies in our laboratory are trying to use an intestinal epithelial cell line, Mode-K cells, to study the change of detoxification genes induced by *H. bilis* stimulation. However, it is unknown whether the TLR4 defect in Mode-K cells from C3H/Hej mice will have an influence on expression of detoxification genes. One limitation of the study on detoxification genes is that there are no monoclonal antibodies available for many of these genes. If available, immunohistochemistry for those detoxification-associated proteins would provide a good tool to locate detoxification molecules.

In addition, the temporal gene expression changes of detoxification genes in different models of intestinal inflammation would be useful. These might include the determination of the expression levels of detoxification genes at early (e.g., 5 days after *H .bilis* colonization) or later (12 weeks after *H. bilis* colonization) and determination of the expression levels of genes in acute versus restitution period of DSS colitis or different time points after B. *hyodysenteriae* infection. These temporal studies would identify what time points and intestinal inflammation models affect most or are most affected by the detoxification processes.

What toxins do the detoxification genes work on? Since the gut harbor huge amount of bacteria and antigens, it would be difficult to determine which specific toxin or deleterious antigen might be a major player in the detoxification process. However, one interesting point for *H. bilis* is that this bacterium has a specific toxin, cytolethal distending toxin (CDT). CDT has the potential to induce distention in cells and to arrest cell division in the G2/M phase of cell cycle.[39] CDT has also been shown to play a key immunomodulatory role that facilitates persistent colonization of *H. hepaticus* and that, in IL-10$^{-/-}$mice, this alteration of the host immune response results in the development of colitis.[40] The possible association between CDT and detoxification genes will be of interest. The study on the CDT mutant *H. bilis* will provide insights on this question.

In conclusion, dysregulation of detoxification genes was observed in multiple models of intestinal inflammation including *Helicobacter bilis* colonization and potentially contribute to the initation or perpetuation of IBD.

Activation of adaptive immunity

Two microarray studies on *Helicobacter bilis* were described in this dissertation. The first study on mucosal gene expression profiles at 15, 30 and 45 DPI after *H. bilis* colonization identified the downregulation of detoxification genes, with the highest fold change at 15 DPI. In the second study, the comparison of mucosal gene expression profiles between mice colonized with *H. bilis* 6 weeks prior to DSS treatment and mice treated with DSS alone were performed. This comparison should address the effects of *H. bilis*. In contrast to the first study, we identified a series of genes associated with activation and recruitment of immune cells.

The discrepancy between two microarray studies might be associated with the microarray techniques and statistic analysis. The much higher fold change of mucosal gene expression at 15 DPI vs. 30 and 45 DPI might skew the differentially expressed genes identified by microarray (false discovery rate ≤ 0.05 and fold change ≥ 2) into the mainly genes associated with 15 DPI. Actually, the relative loose statistic criteria (e.g., false discovery rate <0.1) will identify many more genes associated with immune responses such as cytokine and chemokine (data not shown). In addition, the downregulation of detoxification genes was confirmed by qRT-PCR in mice colonized with *H. bilis* plus treated with DSS in comparison to mice treated with DSS alone. Another possible contributor for this discrepancy is that ceca tissues were used in the first microarray study while colon tissues were used in second microarray study.

Our microarray study and cell infiltration analysis demonstrated that *H. bilis* colonization could induce the activation and recruitment of T cells, B cells and macrophages. However, the roles of these cell types in this special intestinal inflammation (i.e., *H. bilis* colonization plus low dose DSS treatment in C3H gnotobiotic mice) are still unknown.

Th17 T cells have been shown to play essential role in several IBD models and autoimmune diseases.[41] The upregulation of IL17 gene expression in lamina propria lymphocytes and also mucosa levels indicate the potential importance of Th17 T cells in the development of colitis in this model. Adoptive transfer of T cells into SCID or $Rag^{-/-}$ mice would be able to determine the importance of pathogenic T cells in the development of colitis.

However, we should be cautious that other T or non-T cells might also express or secret IL17. Intracellular staining for IL17 might be needed to test it. As for B cells, the increased antibody responses to commensal bacteria after *H. bilis* colonization were observed. The current paradigm suggests that immune responses to commensal bacteria play an essential role in the IBD development.[4] It will be of interest to study how these antibody responses to commensal bacteria contribute to colitis development. The macrophages are essential cells for DSS colitis, which was shown by the induction of DSS colitis in SCID mice with rare lymphocytes. Interestingly, *H. bilis* also has been shown to induce colitis in SCID mice.

One of critical questions in our intestinal inflammation model (*H. bilis* + DSS) is the association between downregulation of detoxification genes and activation/recruitment of immune cells. It will be of interest to study whether the one factor leads to the other. In SAMP1/Yit mice -A Crohn's disease model, bone marrow chimera experiments showed that increased permeability of epithelial cells, rather than activation of hematopoietic cells, was primary defect and would lead to the activation of T cells and development of the ileitis.[42]

Low dose DSS provides an additional stimulus to host colonized with *H. bilis*, leading to the development of colitis. DSS is widely considered to have direct toxic effects on intestinal epithelial cells.[43] Our preliminary data suggest the DSS treatment downregulated the expression of genes associated with cell cycle, indicating that epithelial cells might be less proliferative, thus affecting intestinal barrier functions (data not shown). BrdU staining for epithelial cells might provide more evidences on how DSS affect the epithelial cell proliferation. In addition, DSS treatment has been shown to downregulate the detoxification-associated genes.

Hypothesis for intestinal inflammation models (H. bilis colonization + low dose DSS)

From the above discussions, we hypothesize that *H. bilis* and DSS play synergetic and yet distinct roles in the development of colitis.

Both *Helicobacter bilis* and low dose of DSS might affect the detoxification ability of intestinal epithelial cells. However, DSS might also exert direct apoptotic effects on epithelial cells while *H. bilis* itself seems to be a potent stimulator to T cells, B cells or macrophages in lamina propria.

Single treatment with either *H. bilis* or low dose DSS is not be potent enough to initiate colitis development. However, co-treatment of *H. bilis* and low dose DSS may damage the integrity or barrier functions of intestinal epithelial cells. The alteration of epithelial integrity or barrier functions allows *H. bilis* and commensal bacteria to access to lamina propria, to activate various immune cells, eventually leading to intestinal inflammation. The requirement of both *H. bilis* and DSS for the development of colitis is consistent with "Multiple-hit hypothesis". [44]

Hypoxoside, Brachyspira hyodysenteriae, DSS and NF-kB signaling pathway

In this dissertation, hypoxoside, an anti-inflammatory drug, has been shown to ameliorate the acute colitis induced by *Brachyspira hyodysenteriae*. More importantly, we showed that hypoxoside could downreglate the expression levels of genes associated with NF-κB signaling pathway and reduced the mucosal protein levels of p65 subunit.

Given the fact that many pro-inflammatory cytokines and chemokines are regulated by transcriptional factor NF-κB, it is expected that hypoxoside can reduce the intestinal inflammation through the downregulation of NF-κB signaling pathways.

To further test the therapeutic effects of hypoxoside, we tested if oral treatment of hypoxoside could ameliorate or prevent DSS-induced colitis at the dose of 1.25% for five days. Surprisingly, it was found that hypxoside treatment did not prevent or ameliorate the DSS-induced colitis, as shown by no significant change in histological lesions. In addition, mice treated with DSS and hypoxoside groups had more intestinal bleeding in comparison to mice treated with DSS alone, indicating hypoxoside might have deleterious effect on tissue repair (data not shown).

The mechanisms of deleterious effects of hypoxoside on DSS colitis are still unknown. However, accumulating evidence also suggestes NF-κB might have protective effects against colonic insult, especially on intestinal epithelial cells.[45-48] We observed that hypoxoside treatment could reduce the proliferation of epithelial cells. Since DSS might exert direct toxic effect and/or induce epithelial apoptosis, the co-treatment of hypoxoside and DSS is likely to greatly affect the epithelial cell turnover, thus damaging the tissue repair. In addition, hypoxoside has been shown *in vitro* to inhibit cyclooxygenase-2 (Cox-2) and

cyclooxygenase-1 (Cox-1), [20] which are essential for synthesis of cytoprotective prostaglandins in intestine. However, controversial evidence on the effects of hypoxoside on Cox-1 and Cox2 also exist.[49]

Similarly and interestingly, another plant extract and anti-inflammatory drug, luteolin, was shown to ameliorate the colitis in $IL10^{-/-}$ mice, yet aggravated DSS-induced experimental colitis.[50] More importantly, luteolin treatment significantly enhanced intestinal epithelial cell pro-apoptotic caspase-3 activation and prevented the induction of NF-κB-Dependent cytoprotective molecules such as COX-2 following DSS-exposure.[50]

It was proposed that, in the context of a primarily intact epithelial layer, antigen- (e.g. bacteria-) induced NF-κB activation in lamina propria mononuclear cells leads to NF-κB dependent pro-inflammatory cytokine secretion, thus its inhibition by luteolin likely ameliorates intestinal inflammation.[50, 51] NF-κB activation in intestinal epithelial cells is likely protective (wound-healing response, anti-apoptotis), and its inhibition by luteolin consequently aggravates intestinal damage by DSS.[50, 51] The NF-κB function in intestine was illustrated in supplemental figure 1.[51] Further investigations are needed to test if hypoxoside have the similar mechanisms on intestinal inflammation.

Conclusion

The results in this dissertation have suggested some possible mechanisms how different agents as demonstrated by a mild pathogen *Helicobacter bilis* and an anti-inflammatory drug hypoxoside modulate the gene expression profiles and alter the mucosal functions (e.g., epithelial barrier functions and/or immune system in lamina propria), which might contribute to the initiation, perpetuation or amelioration of IBD development. For instance, *Helicobacter bilis* might affect epithelial detoxification and activate the T cells, B cells or macrophages in lamina propria, thus increase the host susceptibility to colitis. On the other hand, hypoxoside could downregulate the NF-κB pathway and ameliorated *Brachyspira hyodysenteriae*-induced colitis. These studies in this dissertation identified some promising gene and signaling targets for IBD such as genes associated with detoxification, activation/recruitment of immune cells and activation of NF-κB pathways and might potentially contribute to the therapeutic intervention of IBD patients. Further studies are

needed to dissect the individual functions of intestinal epithelial cells and lamina propria immune cells.

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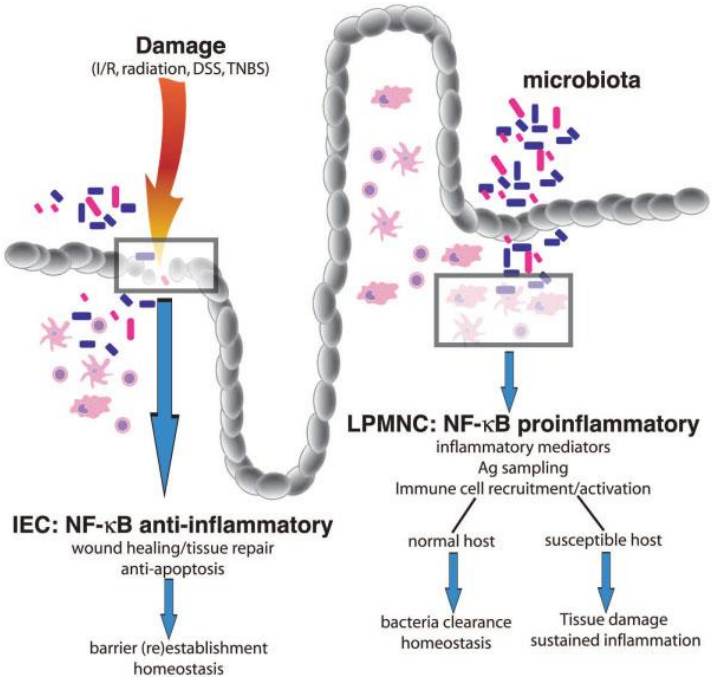

Supplemental Figure 1. Proposed functions of NF-κB in the intestine. [51]Intestinal epithelial cells provide a physical barrier isolating luminal microbiota from a rich mucosal immune system in the lamina propria. Sustained and prolonged epithelial damage compromises host integrity by allowing uncontrolled bacterial translocation, followed by the activation of lamina propria mononuclear cells (LPMNC) such as macrophages, dendritic cells, and T and B lymphocytes. TLR/ NF-κB signaling in intestinal epithelial cells has a protective function by inducing the transcription of genes involved in wound-healing, tissue repair, and intestinal epithelial cell survival, thereby contributing to barrier reestablishment. Blocking NF-κB activity in this biological setting consequently jeopardizes intestinal homeostasis (left). TLR/ NF-κB signaling in LPMNC participates in antigen sampling, the recruitment and activation of immune cells, which in the normal host results in bacterial clearance and reestablishment of host homeostasis. In a colitogenic susceptible host, TLR/ NF-κB signaling derived from LPMNC contributes to the chronic inflammatory process and tissue damage through the sustained production of proinflammatory and chemoattractive mediators (e.g., cytokines, chemokines, nitric oxide, growth factors, etc.). Blocking NF-κB activity in this biological setting can attenuate intestinal inflammation (right).

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